Thesis for the degree
of Candidatus Scientiarum
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Automated extraction and clean-up of human serum samples for determination of halogenated organic pollutants

DEPARTMENT OF CHEMISTRY
FACULTY OF MATHEMATICS
AND NATURAL SCIENCES
UNIVERSITY OF OSLO 05/2005

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ACKNOWLEDGEMENTS

The work presented in this thesis has been performed at the National Institute of Public Health, Division of Environmental Medicine, Department of Analytical Chemistry in the time period 2003 to 2005.

I want to thank my supervisors **Georg Becher**, head of Department of Analytical Chemistry and **Cathrine Thomsen** for giving me this assignment, for good help and interesting discussions.

I am especially grateful to **all the personnel** at the Department of Analytical Chemistry for priceless help and for creating an enjoyable atmosphere. I would especially like to thank **May Frøshaug** for many fruitful discussions and help in the daily laboratory work, and **Sharon L M Broadwell** for correcting my English.

I am much grateful to all my former **colleagues at "vaksineavdelingen"** who focused my interest on analytical chemistry and encouraged me to pursuit further studies. I would especially like to thank **Bente Møgster** and **Tove Eidem** for all their support through my years at the university.

Most of all I would like to thank my family. My husband **Bjørn,** for support and care through these years. My father, for his interest in my work and studies, and my mother for her never-ending love and encouragement (you will always have a special place in my heart).

National Institute of Public Health, 2005

Veronica H. Liane

Abstract

An analytical method comprised of automated solid phase extraction and determination using gas chromatography electron capture mass spectrometry has been developed for the determination of 12 polybrominated diphenyl ethers (PBDEs), 26 polychlorinated biphenyls (PCBs), two organochlorine compounds (OCs) and two brominated phenols in human serum. The analytes are extracted using a sorbent of polystyrene-divinylbenzene and an additional clean-up is performed on a sulphuric acid silica column to remove lipids.

The method has been validated by spiking horse serum at five levels in the range of 1.2-120 pg PBDEs/g serum, (12-1200 pg BDE-209/g serum), 1.2-120 pg pentabromophenol (PeBP)/g serum, 2.4-240 pg tetrabromobisphenol-A (TBBP-A)/g serum and 3-300 pg PCBs/g serum. Different internal standards were evaluated for the PBDEs. The accuracy given as recovery relative to internal standards, was from 64-150% with relative standard deviations (RSD) ranging from 0.6-56% for the PBDEs. The mean accuracy was 95% and the mean RSD 6.9%. The accuracy for the PCBs was in the range of 29-127% with RSD in the range of 0.3-77%, and with mean accuracy and RSD of 99% and 8.7% respectively. The two OCs hexachlorobenzene and octachlorostyrene had accuracies in the range of 62-104% with RSD in the range of 3.2-15%, the mean accuracy was 93% and the mean RSD 7.5%. The accuracy for the determination of PeBP and TBBP-A were in the range of 77-132% with RSD from 4.5-24%, the mean accuracy and RSD was 109% and 15% respectively. In summary, the overall accuracy and RSD was about 98% and 8.4% respectively. Estimated limits of detection (LOD, signal to noise ratio =3) were in the range of 0.2-1.8 pg/g serum for the PBDEs and phenols, the PCBs and OCs had LODs in the range of 0.1-56 pg/g serum. The method linearity was determined by plotting the concentration of persistent organic pollutants found in the spiked validation samples against the concentration added. Correlation coefficients were in the range of 0.9981-1.0000.

The validated method has been used to investigate the levels of PBDEs and PCBs in pooled serum samples from men (age 40-50 years) sampled in the time period 1977 to 2003, and in pooled serum samples from different age groups sampled in 2002 (all from the general Norwegian population). The sum of seven PBDE congeners (IUPAC No. 28, 47, 99, 100, 153, 154 and 183) has increased from 1977 (0.61 ng/g lipids) to 1998 (4.85 ng/g lipids). From 1999 to 2003 the concentration of PBDEs seems to have stabilized. However, the congener

BDE-153 was found to increase throughout the entire period. On the other hand the sum of five PCBs (IUPAC No. 101, 118, 138, 153 and 180) have decreased steadily from 1977 (665 ng/g lipids) to 2003 (176 ng/g lipids). A comparison was made between the main congeners from PCBs and PBDEs, and it was found that the level of CB-153 was about 46 times higher than the level of BDE-47 in the serum pool from 2003.

In samples from 2002 an increase with age was observed for the PCBs. The highest levels were observed in the age group >60 years where the sum of five PCBs were 308 ng/g lipids and 353 ng/g lipids for women and men, respectively. For the PBDEs the exact opposite trend was observed, with the highest levels in the lowest age group (0-4 years), where the sum of seven PBDEs was 10.4 ng/g lipids. TBBP-A and BDE-209 were detected in almost all samples, but no similar trends to that seen for the PBDEs and PCBs were observed for these compounds.

Abbreviations

ASPEC Automated solid-phase extraction
BB Brominated biphenyl
BDE Brominated diphenyl ether
BFR Brominated flame retardants
CB Chlorinated biphenyl
CI Chemical ionization
CtriBBP-A Chlorotribromobisphenol-A
ECD Electron capture detector
ECMS Electron capture mass spectrometry
ECNI Electron capture negative ionization
EI Electron ionization
FR Flame retardants
GC Gas chromatography
HLB Hydrophilic-lipophilic balance
HRMS High resolution mass spectrometry
I.D. Internal diameter
ISTD Internal standard
LLE Liquid-liquid extraction
LOD Limit of detection
MS Mass spectrometry
MSD Mass spectrometric detector
OCs Organochlorine compounds
PBDE Polybrominated diphenyl ether
PCB Polychlorinated biphenyl
POPs Persistent organic pollutants
PeBP Pentabromophenol
PS-DVB Polystyrene-divinylbenzene
S/N Signal to noise ratio
SPE Solid-phase extraction
TBBP-A Tetrabromobisphenol-A
TBCr Tetrabromo-*o*-cresol
TriBP 2,4,6-Tribromophenol

Keywords

Automated solid phase extraction
Flame retardants
Human serum
On-column lipid decomposition
Persistent organic pollutants
Polybrominated biphenyls
Polybrominated diphenyl ethers
Tetrabromobisphenol-A
Time trend study

1. Introduction

Persistent organic pollutants (POPs) such as brominated flame retardants (BFRs) and polychlorinated biphenyls (PCBs) are now ubiquitous environmental pollutants. The fact that these compounds are persistent means that there is a risk that they remain in an organism or its environment for a long time which may cause long time exposure. To which degree the organic compounds bioaccumulate depends on their solubility in lipids (fats, oils etc.). Halogenation of an organic compound reduces its solubility in water and increases its solubility in lipids.

Flame retardants (FR) are chemicals that are added to materials during or after manufacture, to prevent or reduce the development of a fire. Brominated flame retardants (BFRs) represent 39% of the worldwide market of flame retardants [1]. Other flame retardants include: chlorinated, phosphorous-containing, nitrogen-containing and inorganic flame retardants. BFRs are considered to be highly efficient as FRs and economically advantageous both regarding production and use.

Worldwide, the total production of flame retardants was estimated to be 600 000 tonnes in 1992 [2]. The total market demand of brominated flame retardants in 2001 was 203 790 tonnes, where tetrabromobisphenol-A (TBBP-A) is produced in the largest amounts (119 700 tonnes), hexabromocyclododecane (HBCD) in 16 700 tonnes and Polybrominated diphenyl ethers (PBDEs) in 67 390 tonnes [1].

There is no domestic production of BFRs in Norway. Accordingly, all BFRs in use are imported, as part of flame retarded products or as the chemical itself.

PBDEs are a class of chemicals with the general structure shown in Figure 1. There are theoretically 209 PBDE congeners, with one to ten bromine substituents. The congeners are numbered from 1 to 209 using the system already existing for PCBs [3], instead of using the standard IUPAC nomenclature.

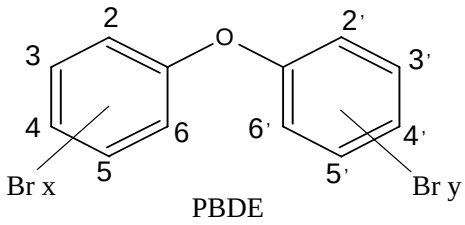

Figure1. The general structure of the PBDEs.

PBDEs are lipophilic substances with log K_{OW} of approximately 4-9. For example 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) has an estimated log K_{OW} of 6.8 while the heptabrominated congener, BDE-183 has a log K_{OW} of 8.3 [4].

PBDEs are commercially available as three products, two of which are mixtures of several congeners. The so-called penta product contains BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154. The octa product contains several hexa-to nona-brominated congeners, and the deca product is almost entirely composed of BDE-209 [5]. In Norway the use of penta- and octa-BDE was prohibited from 1st July, 2004 [6]. Risk assessments are also being made for deca-BDE.

In addition to the PBDEs, several brominated phenolic compounds have been used as flame retardants. TBBP-A is the most widely used of these compounds, 2,4,6-tribromophenol (TriPB) and pentabromophenol (PeBP) are used to a lesser extent (Figure 2). The phenolic BFRs differ from the other BFRs by its hydroxyl groups, which makes them weak acids. TBBP-A has proposed log K_{OW} 4.5-5.3, and are thus more soluble in water than the PBDEs [7].

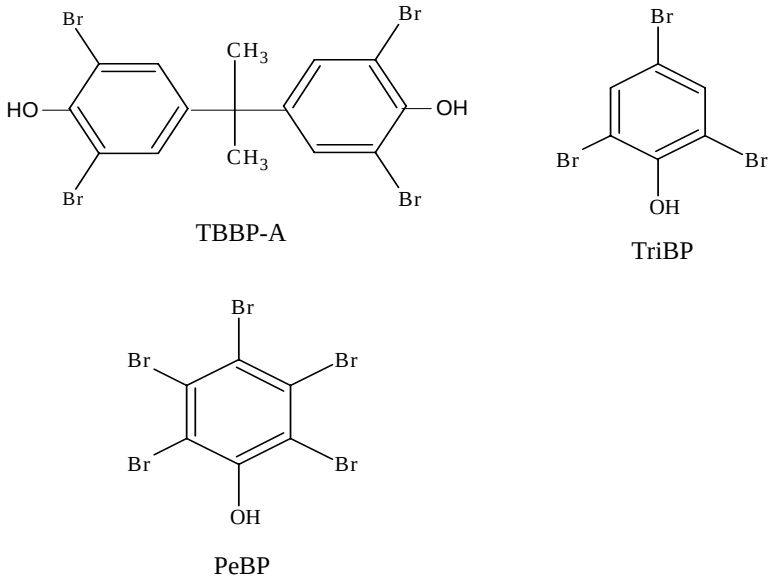

Figure 2. Structures of brominated phenolic compounds.

Flame retardants may be either chemically bound to (reactive) or mixed with (additive) polymeric materials. TBBP-A is used both reactively (90%) and additively, while the PBDEs are only used additively due to the lack of binding sites in the molecules. The primary use of TBBP-A is as a reactive intermediate in the manufacture of flame-retarded epoxy and polycarbonate resins, accounting for approximately 90% of all TBBP-A used. A principal use of TBBP-A epoxy resins is in printed circuit boards. PBDEs are incorporated in acrylonitrile-butadiene-styrene (ABS), high-impact polystyrene (HIPS), flexible polyurethane foams and textile coatings. TriBP and PeBP are used in epoxy resins, phenolic resins and as intermediates for polyester resins [7]. These different types of flame retarded polymer products are present in a number of consumer products, such as computers, household appliances, electronics and TV-sets. PBDEs are also incorporated into products as paints, circuit boards, floor mats, carpets and furniture's.

PCBs shown in Figure 3, have been used commercially since 1930 as dielectric and heat-exchange fluids and in a variety of other applications [8]. As for the PBDEs, 209 congeners are possible, but only 130 are likely to occur in commercial products [8]. In recent years, the production of PCBs have been phased out [8]. Current sources of PCB release include volatilization from landfills, sewage sludge, spills, dredge spoils and improper (or illegal) disposal. The PCBs have log k_{OW}s in the range of 4.5-8.2 with the most chlorinated compounds having the highest values.

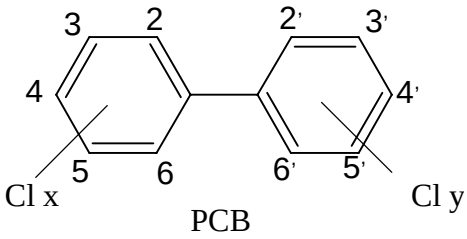

Figure 3. General structure of PCBs.

For humans the main exposure to POPs is through the diet, especially from intake of fatty food of animal origin such as fatty fish. Exposure through air might also show out to be an important exposure path for the PBDEs [9].

A large number of human samples have been analyzed for BFRs and PCBs. PCBs and PBDEs have been found in blood [10-12], adipose tissue [13;14], and in breast milk [15-17]. A study of PBDEs in breast milk from Swedish women show an increase in the PBDE levels from 1972 to 1998, after 1998 the levels decreased up to year 2001 [18]. In serum increasing levels of PBDEs have been reported [19], while the levels of PCBs have been reported to decrease over the years [20;21]. The levels of PBDEs and PCBs in humans and the environment have been reviewed [8;22-24]. BFRs have been studied in blood samples collected from people in different occupational settings [25;26]. Several PBDEs and TBBP-A have also been detected in indoor air at a computer dismantling plant, in rooms containing computers and other electrical equipment and at a factory for assembly of circuit boards [27]. TriBP has been detected in exhaust from vehicles using leaded gasoline [28]. PBDEs, TBBP-A and TriBP have also been detected in laboratory air [29].

PCBs, TBBP-A and PBDEs have also been found in sewage sludge and sediments from a number of locations [30-32]. In a study near a Swedish plastics industry using TBBP-A, higher levels were found in sediment samples collected downstream than upstream, indicating that the plastics industry was the source for these substances [33].

One of the first reports on PBDEs in the environment were published 1981 where these compounds were detected in fish from Sweden [34]. The fish was collected from the Viskan-Klosterfjorden water system, and the dominating congeners found was tetra-BDE followed by

penta and hexa-BDE. Now PBDEs and PCBs are frequently detected in biota such as in fish [35-38], bird eggs [39-41], marine and terrestrial mammals and mussels [42].

It has been shown that the acute toxicity of TBBP-A and PBDEs is low [5;7]. Limited data are available on the toxicity of PBDEs and TBBP-A in humans. In exposure studies of TBBP-A and deca-BDE no evidence for skin irritation or sensitization was found [5;7]. Skin raches have been reported after acute exposure to PCBs [8]. Several PBDEs and PCBs have been found to interact with the aryl hydrocarbon (Ah) receptor, mainly as an antagonists, but also as agonists [43]. Estrogenic and antiestrogenic activity of several PBDEs and TBBP-A have also been reported [44]. In the investigation of nevrotoxic effects of PCBs, PBDEs and TBBP-A it was found that mice neonatally exposed to BDE-47, BDE-99 and several PCB congeners displayed behavioural changes [45]. Exposure of TBBP-A in the same dose range as the PBDEs did not cause any significant changes in behavioural variables investigated. PCBs and PBDEs have been shown to effect the thyroid hormone system [46;47]. Thus both PCBs and PBDEs have the potential to cause negative effects on human health.

Analytical methods used for determination of PBDEs and PCBs are very similar. The separation from biological and environmental matrix is typically performed by extraction with organic solvents as in liquid-liquid extraction (LLE) or more recently by solid-phase extraction (SPE) [12;48]. Chromatography (e.g. gel permeation, silica gel, floricil, activated carbon) is often used to remove matrix components. Lipids can be removed by destructive methods such as treatment with concentrated sulphuric acid, or by non-destructive methods such as gel permeation. PCBs are usually extracted from blood or serum by solvent extraction techniques using hexane, benzene or mixed solvents such as hexane/ethyl ether [49]. BFR have been extracted with 2-propanol/hexane, methylene chloride, hexane, formic acid/2-propanol/water, and hexane/methyl t-butyl ether [49]. Polystyrene-divinylbenzene (PS-DVB) based SPE columns have successfully been used for the extraction of BFRs from breast milk and serum [48]. The separation and quantification of BFRs and PCBs are most often accomplished by gas chromatographic (GC) techniques. Long capillary columns are normally used, but the higher brominated PBDEs have longer retention times and are often separated on shorter columns. The most common approach for identification and quantification is to use electron capture detection (ECD) or mass spectrometric (MS) techniques, such as electron ionization (EI) or electron capture negative ionization (ECNI), or to use high resolution mass

spectrometry (HRMS). The most frequently used injection techniques are splitless and on-column injection [50].

The main objective of this study was to develop a fast, selective and sensitive method for determination of halogenated organic pollutants in human serum, by modifying a previously described method based on SPE and on-column lipid decomposition [12;51], and transferring it to an automated unit. The second objective was to use this method to study the temporal trends and the role of age of POPs in pooled serum samples from the general Norwegian population.

2. Experimental

2.1 Materials and reagents

2,4,4´-Tribromodiphenyl ether (BDE-28), 3,4,4´-tribromodiphenyl ether (BDE-37), 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47), 2,2´,3,4,4´-pentabromodiphenyl ether (BDE-85), 2,2´,4,4´,5-pentabromodiphenyl ether (BDE-99), 2,3´,4,4´,6-pentabromodiphenyl ether (BDE-119), 2,2´,4,4´,5,5´-hexabromodiphenyl ether (BDE-153), 2,2´,4,4´,5,6´-hexabromodipenyl ether (BDE-154), 2,2´,3,4,4´,5,6-heptabromodiphenyl ether (BDE-181), 2,2´,3,4,4´,5´,6-heptabromodipenyl ether (BDE-183), 2,2´,3,3´,4,4´,5,5´,6,6´-decabromodiphenyl ether (BDE-209), ^{13}C-2,4,4´-trichlorobipenyl (CB-28), ^{13}C-2,2´,5,5´-tetrachlorobiphenyl (CB-52), ^{13}C-2,2´,4,5,5´-pentachlorobiphenyl (CB-101), ^{13}C-2´,3,4,4´,5-pentachlorobipenyl (CB-123), ^{13}C-2,3´,4,4´,5-pentachlorobiphenyl (CB-118), ^{13}C-2,3,4,4´,5-pentachlorobiphenyl (CB-114), ^{13}C-2,2´,4,4´,5,5´-hexachlorobipenyl (CB-153), ^{13}C-2,3,3´,4,4´-pentachlorobiphenyl (CB-105), ^{13}C-2,2´,3,4,4´,5´-hexachlorobiphenyl (CB-138), ^{13}C-2,3´,4,4´,5,5´-hexachlorobiphenyl (CB-167), ^{13}C-2,2´,3,4,4´,5,5´-heptachlorobiphenyl (CB-180), ^{13}C-2,3,3´,4,4´,5-hexachlorobiphenyl (CB-156), ^{13}C-2,3,3´,4,4´,5´-hexachlorobiphenyl (CB-157), ^{13}C-2,2´,3,3´,4,4´,5-heptachlorobiphenyl (CB-170), ^{13}C-2,3,3´,4,4´,5,5´-heptachlorobiphenyl (CB-189), ^{13}C-2,2´,3,3´,4,4´,5,5´-octachlorobiphenyl (CB-194), ^{13}C-2,2´,3,3´,4,4´,5,5´,6,6´-decachlorobiphenyl (CB-209), hexachlorobenzene (HCB), ^{13}C-hexachlorobenzene and octachlorostyrene (OCS) and tetrabromobisphenol-A (TBBP-A) were obtained from CIL (Andover, MA). 2,3,3´,4,4´,5-hexabromodiphenyl ether (BDE-156), 3,3´,4,4´-tetrabromodiphenyl ether (BDE-77), 2,2´,4,4´,6-pentabromodiphenyl ether (BDE-100) and ^{13}C-2,2´,3,3´,4,4´,5,5´,6,6´-deca bromodiphenyl ether (BDE-209) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). 2,2´,5-tribromodiphenyl ether (BDE-18), 2,2´,4,6´-tetrabromodiphenyl ether (BDE-51), 2,2´,4,5´,6-pentabromodiphenyl ether (BDE-103), 2,2´,3,4,4´,5´-hexabromodiphenyl (BDE-138), 2,2´,5-trichlorobiphenyl (CB-18), 2,4,4´-trichlorobipenyl (CB-28), 2,2´,5,5´-tetrachlorobiphenyl (CB-52), 2,4,4´,5-tetrachlorobiphenyl (CB-74), 2,3´,4,4´-tetrachlorobipenyl (CB-66), 3,4,4´,5-tetrachlorobiphenyl (CB-81), 2,2´,4,4´,5-pentachlorobiphenyl (CB-99), 2,2´,4,5,5´-pentachlorobiphenyl (CB-101), 2,3,3´,4,4´-pentachlorobiphenyl (CB-105), 2,3,3´,4´,6-pentachlorobipheny (CB-110), 2,3,4,4´,5-pentachlorobiphenyl (CB-114), 2,3´,4,4´,5-pentachlorobiphenyl (CB-118), 2´,3,4,4´,5-pentachlorobipenyl (CB-123), 2,2´,3,3´,4,4´-hexachlorobiphenyl (CB-128), 2,2´,3,4,4´,5´-hexachlorobiphenyl (CB-138), 2,2´,4,4´,5,5´-hexachlorobipenyl (CB-153),

2,3,3´,4,4´,5-hexachlorobiphenyl (CB-156), 2,3,3´,4,4´,5´-hexachlorobiphenyl (CB-157), 2,3´,4,4´,5,5´-hexachlorobiphenyl (CB-167), 2,2´,3,3´,4,4´,5-heptachlorobiphenyl (CB-170), 2,2´,3,4,4´,5,5´-heptachlorobiphenyl (CB-180), 2,2´,3,4,4´,5´,6-heptachlorobiphenyl (CB-183), 2,2´,3,4´,5,5´,6 (CB-187), 2,3,3´,4,4´,5,5´-heptachlorobiphenyl (CB-189), 2,2´,3,3´,4,4´,5,5´-octachlorobiphenyl (CB-194), 2,2´,3,3´,4,4´,5,6,6´-nonachlorobiphenyl (CB-207) and 2,2´,3,3´,4,4´,5,5´,6,6´-decachlorobiphenyl (CB-209) were purchased from AccuStandard Inc. (New Haven, CT). TriBP and tetrabromo-*o*-cresol (TBCr) were obtained from Aldrich (Milwaukee, WI) and PeBP from Acros (Geel, Belgium). Chlorotribromobisphenol-A (CtriBBP-A) was a gift from the Wallenberg Laboratory (University of Stockholm, Sweden).

All solvents were of pesticide grade, and were purchased from LabScan (Dublin, Ireland). Sulphuric acid (H_2SO_4) and formic acid of analytical grade, Silica gel 60 (0.063-0.200 mm) for column chromatography and sodium sulphate (Na_2SO_4) for organic trace analysis was obtained from Merck (Darmstadt, Germany). N-methyl-N-nitroso-p-toluenesulfonamide (Diazald) was purchased from Aldrich (Milwaukee, WI). Water was purified using an Elga Option 4 Water Purifier device (Elga, Bucks, UK).

All glassware was washed in 2.5% RBS 25 foaming cleaner (Chemical Products, Brussels, Belgium) rinsed with distilled water, and subsequently heated at 450°C for 4 h (volumetric equipment was not heated).

2.1.1 SPE columns and equipment

Oasis® HLB custom-made solid phase extraction cartridges (540 mg/ 3 mL) were purchased from Waters Corporation (Milford, MA). For preliminary method development, Isolute 101 (200 mg/ 3 mL) obtained from International Sorbent Technology (Mid Glamorgan, UK) and Strata-X (60 mg/ 3 mL) from Phenomenex (Torrance, CA) was used.

2.2 Samples

2.2.1 Validation samples

For method validation horse serum from Sigma-Aldrich (H-1270) was used. The serum was homogenized by sonication in a Branson 2510E-MT ultrasonic cleaner for 5 minutes. The serum was then divided into ten sub-samples of 50 mL each, and stored at -18°C until analysis. As procedural blanks five mL of purified, sterilized water containing 0.9% sodium chloride was used.

2.2.2 Sample pools from Norway

A study was performed on two series of pooled serum samples from a local bio-bank at the National Institute of Public Health. The serum had been sampled from different county hospitals in Norway and stored at - 20°C. The first series (later referred to as the time-trend study) was restricted to men in the age group 40-50 years, to limit variation of body burden with gender and age. Five hundred μL of serum from each individual was pooled and stored at - 20°C. Serum samples from 2002 were chosen for study on BFRs and PCBs levels in groups with different age and gender (later referred to as the age-trend study). In the serum bank the individuals had been divided into eight age groups, serum from 20 individuals was pooled in each age group. For the 0-4 years old where limited sample volume was available, only 250 μL was taken from each individual, 29 individuals in total. In the 1997 pool, serum from 14 individuals was sampled because of limited sample volume available from this year. Details concerning the sample pools are listed in Table D1 in appendix. In addition five sample pools prepared earlier was incorporated in the study (1977, 1982, 1988, 1991 and 1994).

2.2.3 Control samples

As control samples for the PCBs, three samples from an interlaboratory comparison study administrated by Institute national de santé publique du Québec, Arctic Monitoring and Assessment Programme (AMAP) was used. The samples were pooled human serum spiked with PCBs at different levels.

2.3 Preparation of standard solutions

Stock solutions and dilutions from stock solutions were made volumetrically. Mixtures of analytes were made at eight levels for PBDEs and phenols in the concentration range of 0.1-40 pg/μL for all PBDEs except BDE-209, which were in the concentration range of 1-400 pg/μL. For the phenols the concentrations were in the range of 0.05-29 pg TriBP/μL, 0.1-58 pg PeBP/μL and 0.2-116 pg TBBP-A/μL. Analyte mixtures of PCBs were made at twelve levels in the concentration range of 0.1-490 pg/μL (0.1-500 pg/μL for mono-ortho PCB). This mixture also contained the following ^{13}C internal standards: HCB, CB-28, CB-52, CB-101, CB-123, CB-118, CB-114, CB-153, CB-105, CB-138, CB-167, CB-180, CB-156, CB-157, CB-170, CB-189, CB-194 and CB-209 with concentration 10 pg/μL for all except HCB which was 15 pg/μL.

The internal standard solution for PBDE consisted of BDE-18, BDE-51, ^{13}C-BDE-77, BDE-103, BDE-156, BDE-181 and ^{13}C-BDE-209 with concentration 2.5 pg/μL for all compounds except ^{13}C-BDE-209 which were 25 pg/μL. Internal standards (ISTDs) for phenols consisted of 1 pg/μL TBCr and 3 pg/μL CtriBBP-A. Two separate solutions of ISTDs were made for PCBs, one for mono-ortho PCBs with concentration 5 pg/μL and the following ^{13}C congeners; CB-105, CB-114, CB-118, CB-123, CB-156, CB-157, CB-167 and CB-189. The other PCB ISTD contained the following ^{13}C congeners: HCB, CB-28, CB-52, CB-101, CB-153, CB-138, CB-180, CB-170, CB-194 and CB-209 with concentration 20 pg/μL. As recovery standard, CB-207 was used with the concentration 8.75 pg/μL.

The BFR standards (PBDEs and phenols) used for calibration were prepared by mixing 30 μL standard solution with 30 μL internal standard solution and 15 μL recovery standard. The PCB standards were prepared by mixing 30 μL standard solution (with ISTDs) with 15 μL recovery standard.

2.4 Instrumentation

2.4.1 ASPEC

The solid phase extraction was performed using an ASPEC XL4 from Gilson (Middleton, WI). ASPEC XL4 is a rapid and robust 4-channel system, each channel being equipped with its own syringe pump and needle. Four 2-way communal solvent ports permit the method to select from 8 solvent-bottles by switching the ports, in addition solvents placed in tubes on the sample tray can be used.

2.4.2 GC-MS

The chromatographic separation was performed on an HP (Avondale, PA) 6890 gas chromatograph equipped with an HP 7683 automatic liquid sampler. The HP ChemStation B.02.05 and D.01.02 software's operated the system. Columns used for separation of the individual components are shown in Table 1.

Table 1. Columns used in analysis POPs.

Compounds	Column[a]	Length (m)	Internal diameter (mm)	Film thickness (μm)
Phenols	DB-5MS	25	0.25	0.25
PBDEs	DB-5MS	25	0.25	0.25
BDE-209	DB-5MS	15	0.25	0.10
PCBs	DB-5MS	60	0.25	0.25

[a] 5% Phenyl 95% dimethyl polysilxane, Agilent Technologies, Inc., CA.

A deactivated retention gap of 1.5 m × 0.32 mm I.D. fused silica (Agilent Technologies, Inc., CA) was used in front of all the columns. Samples of 2 μL were injected at 290°C in pulsed splittless mode with a pulsed pressure of 3.79 bar for 1.5 minutes. Helium (99.998%, Aga) was used as a carrier gas, and the flow was held constant at 1.2 mL/min.

Temperature programs used for the different compounds are listed in Table 2-4.

Table 2. Temperature program for the PCBs.

Step	°C/min	Next °C	Hold min	Runtime
Initial		100	1.0	1.0
Ramp 1	30	190	1.0	5.0
Ramp 2	2.0	250	0.0	35.0
Ramp 3	5.0	300	0.0	45.0
Ramp 4	20	325	1.0	47.25

Table 3. Temperature program for the phenols and PBDEs.

Step	°C/min	Next °C	Hold min	Runtime
Initial		90	1.0	1.0
Ramp 1	20	190	0.0	6.0
Ramp 2	5.0	230	0.0	14.00
Ramp 3	1.0	235	0.0	19.00
Ramp 4	3.5	250	0.0	23.29
Ramp 5	30	325	4.0	29.79

Table 4. Temperature program for BDE-209.

Step	°C/min	Next °C	Hold min	Runtime
Initial		90	1.0	1.0
Ramp 1	20	190	0.0	6.0
Ramp 2	4.0	230	0.0	16.00
Ramp 3	1.0	235	0.0	21.00
Ramp 4	3.5	250	0.0	25.29
Ramp 5	30	325	4.0	31.79

The mass spectrometer, an HP 5973 MSD was operated in electron capture negative ionization mode (ECNI) for the determination of PBDEs, phenols and PCBs, in addition some of the PCBs were determined in electron ionization mode (EI). Methane (99.99%, Aga) was used as buffer gas in the ECNI mode. The parameters applied for the ion source, quadrupole, MS interface and the electron energy are listed in Table 5.

Table 5. MS parameters.

Compounds	Ion source (°C)	Quadrupole (°C)	MS interface (°C)	Electron energy (eV)
PBDEs/Phenols	250	106	300	123.7
BDE-209	200	106	300	119.1
PCBs (CI mode)	150	106	300	128.4
PCBs (EI mode)	230	150	300	69.9

2.4.3 Qualitative identification of BFRs and PCBs

The analytes were identified by comparing their retention time with the respective retention time of the calibration standards. The brominated compounds were monitored at *m/z* 79 and 81, BDE-209 was monitored at *m/z* 484.6 and 486.6. Both ions were used for identification and the abundance ratio was used for confirmation. The PCBs were monitored at the respective masses for their molecular ions: *m/z* 256 (tri-CB), *m/z* 292 (tetra-CB), *m/z* 326 (penta-CB), *m/z* 360 (hexa-CB), *m/z* 394 (hepta-CB), *m/z* 430 (octa-CB), *m/z* 464 (nona-CB) and *m/z* 498 for (deca-CB). The PCBs without ^{13}C-ISTD were monitored at *m/z* 35 and *m/z* 37 in addition to their molecular ions.

2.5 Quantification

All compounds were quantified using peak areas obtained by integration in the HP chemstation software and Internal standard calibration.

2.6 Determination of lipid content

The lipid content of the pooled serum samples was determined at The National Hospital of Norway (Oslo, Norway) according to a method described by Grimvall et al. [52]. Details concerning fat content and number of individuals represented in each pool are summarized in Table D1 in appendix.

2.7 Sample preparation

2.7.1 Homogenization and dilution

The frozen serum samples were thawed in a refrigerator at 4°C overnight and brought to ambient temperature. The samples were then sonicated as described in section 2.2.1 for 5 minutes to ensure that representative sub samples were obtained.

Approximately 5 g of each serum sample were weighed into separate tubes (15 mL with screw cap) and spiked, see appendix section B. The samples were then whirlmixed, sonicated for 5 minutes and placed in a refrigerator overnight to equilibrate. After bringing the samples to ambient temperature and whirlmixing for 5 minutes, 5 mL of formic acid/2-propanol (4+1 v/v) were added to denaturate the proteins. The samples were again sonicated for 5 minutes and stored in a dark place for 50 minutes. Finally 5 mL of 20% 2-propanol was added to each tube to dilute the sample, followed by another 5 minutes of sonication. (The samples were now ready for extraction).

2.7.2 Solid Phase extraction

2.7.2.1 Extraction procedure

The extraction procedure was performed on an ASPEC XL4, see section 2.4.1, and is outlined in Table 6. To eliminate impurities in the column material, the columns were prewashed with methanol and methanol/dichloromethane. The columns were then conditioned with methanol and methanol/water before the diluted sample was applied. The sample tubes were washed with methanol/water, to remove residual serum, which was then added to the columns. The columns were then washed with 2-propanol and dried for 45 minutes with nitrogen (99.999%, Aga), this was done to remove residual water in the columns and thereby making the pores in the packing material fully accessible to the eluent and also prohibiting water in the eluate. Three times during the drying process 20 μL of 70% methanol were added to facilitate extrusion of water from the micropores of the sorbent material. After drying the analytes were eluted with methanol/dichloromethane.

Table 6. SPE procedure.

Step	Process	Application	Flow
1	Prewash	3 mL methanol	1.5 mL/min
2	Prewash	3 mL 30% methanol in dichloromethane	1.5 mL/min
3	Condition	5 mL methanol	1.5 mL/min
4	Condition	5 mL 5% methanol in water	1.5 mL/min
5	Load	15 mL diluted plasma or serum sample	0.4 mL/min
6	Load	5 mL 5% methanol in water	0.4 mL/min
7	Wash	10 mL 5% 2-propanol	1.5 mL/min
8	Dry	1.0 bar N_2 for 45 min.	
9	Elute	12 mL 30% methanol in dichloromethane	0.4 mL/min

2.7.2.2 Optimization of the extraction procedure

During method development several SPE columns were evaluated for extraction of POPs from serum. Different solvents and solvent mixtures were investigated in order to find the optimal eluent. Approximately 5 g of horse serum was spiked with 30 µL of standard solution at an intermediate concentration level, and prepared according to section 2.7.2.2. Lipids were then removed from the sample as described in section 2.7.3, concentration and derivatization was performed according to section 2.7.4.

2.7.3 Additional clean-up (lipid removal)

Prior to the additional clean-up step, the eluates from the SPE columns were evaporated to a few µL at 40°C in a TurboVap LV (Zymark, Hopkinton, MA) under a gentle stream of nitrogen (99.999%, Aga). The residues were redissolved in 3.5 mL n-heptane/dichloromethane (3:1, v/v), and the extracts were subsequently subjected to the clean-up procedure.

2.7.3.1 Preparation of sulphuric-acid silica columns

Silica gel 60 rinsed with methanol and dichloromethane was activated overnight at 130°C. Sodium sulphate was dried at 600°C overnight.

The columns were packed using empty SPE tubes of polyethylene from Sigma-Aldrich (St.Louis, MO). The activated silica was mixed with concentrated sulphuric acid in the ratio 3:1 (w/v), and shaken until a homogenous mixture was obtained. Sulphuric-acid silica and

sodium sulphate were then filled into the empty SPE tubes, creating alternating layers, (Figure 4). Using columns with alternating layers was found to be more efficient than a one-layer column. The sodium sulphate was added for removal of excess water in the sample extracts.

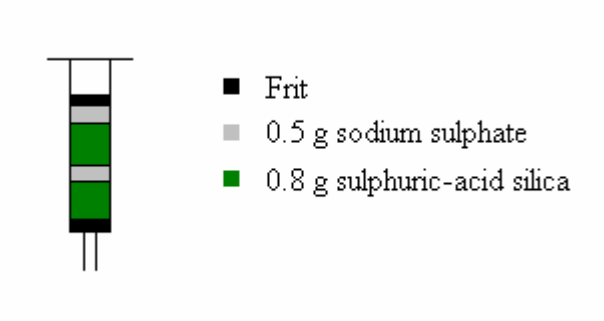

Figure 4. Packed sulphuric-acid silica column.

The additional clean-up step was also performed on the ASPEC XL4 from Gilson.

2.7.3.2 Clean-up procedure

The clean-up procedure is outlined in Table 7. First the columns were washed with heptane to remove air bubbles, and then conditioned with n-heptane/dichloromethane. The redissolved extracts were then applied and the eluate immediately collected in a glass tube. The initial tube was washed with n-heptane/dichloromethane, and then applied to the column. Columns were eluted with another portion of n-heptane/dichloromethane and dried by pushing air through.

Table 7. Clean-up procedure.

Step	Process	Application	Flow
1	Wash	10 mL n-heptane	1.0 mL/min
2	Condition	3.0 mL n-heptan/dichloromethane (3:1, v/v)	1.0 mL/min
3	Load and collect	3.7 mL sample	0.4 mL/min
4	Load and collect	2.2 mL n-heptan/dichloromethane (3:1, v/v)	0.4 mL/min
5	Elute	4.0 mL n-heptan/dichloromethane (3:1, v/v)	0.4 mL/min

2.7.3.3 Optimization of the clean-up procedure

Since the clean-up method is being used for both milk and serum samples it was important to find the optimal conditions were the largest amounts of lipids could be removed, with the limitation being the use of 3 mL SPE tubes. Different bed volumes of sorbent, and strength of the sulphuric acid silica were investigated. The effect of dividing the columns into several layers was also examined. Different amounts of soy oil were dissolved in n-heptane/dichloromethane (3:1, v/v), and then applied to the columns as described in section 2.7.3.2. The eluates were collected in pre-weighed and heated (110°C) beakers and evaporated under a stream of nitrogen. After the evaporation the beakers were weighed and the amount of lipids remaining calculated.

The possible loss of POPs during clean-up was examined by spiking 3.5 mL of n-heptane/dichloromethane (3:1, v/v) with 30 μL of a standard solution and applying it to the columns as described in section 2.7.3.2.

2.7.4 Derivatization

The phenolic compounds were methylated by derivatization of the hydroxy groups. Diazomethane, synthesized by base-catalyzed decomposition of N-methyl-N-nitroso-p-toluensulfonamid (diazald) [53], was used as alkylating reagent.

The sample extracts from the additional clean-up step were concentrated under a gentle stream of nitrogen to a final volume of approximately 30 μL. The extracts were derivatized by adding 25 μL of diazomethane, the samples were then whirlmixed and left in a dark place at ambient temperature for 30 minutes. The excess of derivatization reagent was evaporated by heating at 40°C for approximately 15 minutes (or until the yellow colour disappeared). Finally 15 μL of recovery standard (CB-207) was added, the samples were now ready for GC-MS analysis.

2.8 Method validation

The presented method was validated according to guidelines from Tønseth and Døhl [54] and the International Conference of Harmonisation [55]. Seventeen samples were spiked at five levels in the range of 1.2-120 pg PBDEs/g serum, 12-1200 pg BDE-209/g serum, 1.2-120 pg PeBP/g serum, 2.4-240 pg TBBP-A/g serum and 3-300 pg PCBs/g serum, see Table 9. A solvent exchange was performed on the spiking solutions prior to the sample preparation, to limit the volume of organic solvents (section A, appendix).
Each level was prepared in four replicates except level four which consisted of one replicate.
Four horse serum samples were extracted, and three of them were added ISTDs. The fourth non-spiked sample was used to verify that the horse serum did not contain any native concentrations of the ISTDs. In addition three procedural blanks were extracted. The samples were extracted in random order, in three batches over a period of two weeks.

The intermediate precision was investigated after two months by spiking four horse serum samples at an intermediate concentration level.

The sample extracts were injected once per analysis. Calibration standards were injected three times per sequence, at the beginning, middle and at the end. Calibration curves were made from the calibration standards injected at the beginning and at the end of the sequence.

3. Results and discussion

3.1 Automation

When automating the method and transferring it to the ASPEC XL4 there were some challenges. In the manual SPE method, lipid decomposition was performed directly on the columns with concentrated sulphuric acid, this was not possible on the ASPEC, since tubing and needles were not compatible with sulphuric acid. Some other limitations were the SPE columns that were limited to one and three mL cartridges and the collection tubes, which were limited to 6 mL. When eluting with more than 6 mL several collection tubes must be used, leading to a limitation in the number of samples that can be extracted.

The ASPEC is designed to minimize carry-over. The only liquids that ever come into contact with the syringe pumps are those from the reservoir. The liquids transferred from the sample tray come into contact with the needle and transfer tubing only. Carry-over was investigated by spiking four horse serum samples, these samples were extracted followed by the extraction of four blanks (sterilized water containing 0.9% sodium chloride). The blanks were then analyzed for phenols and PBDEs. The most abundant congeners found in the blanks were BDE-47, BDE-99 and TriBP, these compounds have previously been reported in procedural blanks [29]. The level of these compounds found in this experiment was comparable with levels found in other procedural blanks. A carry-over effect was therefore not found.

Some of the advantages of using an ASPEC are the small consumption of solvents compared to normal LLE and the small need of personnel. Up to 24 samples can be extracted with the SPE method outlined in section 2.7.2.1 within 24 hours. The additional clean-up method outlined in section 2.7.3.2 is less time consuming and 96 samples can be cleaned-up within the same time period.

3.2 Optimization of SPE

Isolute 101 was the first column to be investigated. Isolute 101 is a highly cross-liked PS-DVB copolymer, with spherical particles of 60 μm, pore size of 100 Å and a surface area of approximately 500 m^2/g, (Figure 5). It was found that spiking directly on the columns or in blanks gave a higher yield than spiking in serum, indicating that the spiked compounds were

not fully released from the matrix. The most lipophilic PBDEs were also found to give a poor recovery compared to the less lipophilic PBDEs.

The recovery of POPs in horse serum was improved by performing the sample preparation (weighing, denaturation and dilution) in sample tubes, (instead of Duran flasks), which could be used directly on the ASPEC. By doing this an additional transfer step was eliminated. With the main problem being the poor recovery of the most lipophilic PBDEs, it was investigated if these compounds were lost during sample application or washing of the columns. The strength of the dilution solvent and washing solution was reduced to see if this would prevent a possible breakthrough of the compounds. A 7% 2-propanol solution was used for dilution (previously 20%) and a 5% methanol solution was used for washing (previously 10%). The analytes was eluted with 100% dichloromethane. No improvement was seen for the lipophilic PBDEs, and an overall recovery of about 60% was found.

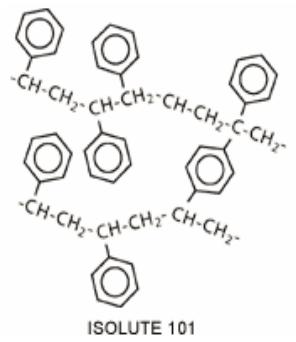

Figure 5. Polystyrene-divinylbenzene copolymer sorbent, from Isolute 101 columns.

Elution solvents and elution volumes was also investigated. The recovery of PBDEs and phenols when eluting with different solvents was examined. The overall mean recovery of PBDEs when eluting with the different solvents was: 77.6% when eluting with 12 mL of 100% dichloromethane, 46.4% when eluting in two steps, first with 6 mL of dichloromethane and then with 6 mL dichloromethane in heptane (1:1, v/v) and 75.7% recovery was found when eluting with 12 mL of toluene (Figure 6). For the phenols the best yields in this experiment was found when eluting with 6 mL dichloromethane and 6 mL dichloromethane in heptane (1:1, v/v), (134.3% TriBP, 96.2% PeBP and 93.5% TBBP-A).

Dichloromethane was found to be a good elution solvent for the PBDEs and PCBs, but to improve the results for the phenols methanol was added to the solution. The methanol will facilitate the elution by interrupting hydrophobic interaction and increasing the solubility of

phenols in the elution solvent. A better yield was not obtained by increasing the elution volume above 12 mL.

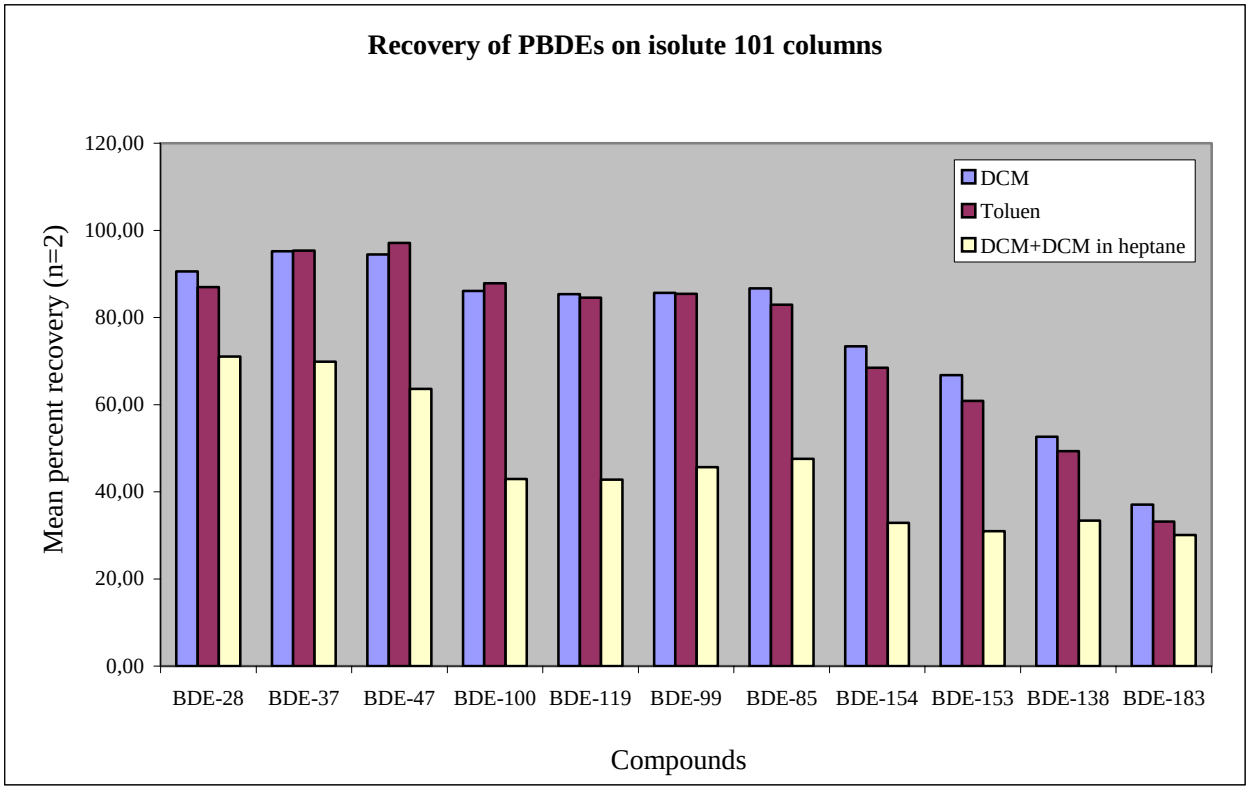

Figure 6. Recovery of PBDEs with different elution solvents performed on isolute 101 columns.

The spiking components were dissolved in organic solvents, which might lead to a breakthrough of analytes on the SPE columns during application. The effect of a solvent exchange from these organic solvents to more polar solvents was investigated and ethanol was found suitable (Appendix section A).

Strata-X and Oasis HLB columns were also examined. Strata-X is a patent pending polymeric sorbent with similar features as Oasis HLB. The Strata-X columns had a particle size of 33 μm, 85 Å pore size and a surface area of 800 m^2/g. Oasis HLB is a hydrophilic-lipophilic water-wettable reversed phase sorbent. It is made from two monomers, the hydrophilic N-vinylpyrrolidone (Figure 7) and the lipophilic divinylbenzene (Figure 5). The N-vinylpyrrolidone, provides an enhanced retention of polar analytes (phenols). The Oasis HLB columns have a surface area of 810 m^2/g, pore size 80 Å and an average particle diameter of 60 μm.

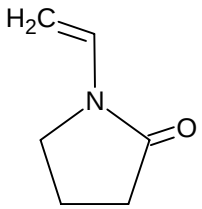

Figure 7. N-Vinylpyrrolidone monomer.

Oasis HLB were purchased from Waters as bulk, and empty 3 mL cartridges were packed with 250, 300 and 500 mg of sorbent and compared with 3 mL Strata-X columns with 500 mg sorbent. The results are shown in Figure 8.

Samples were prepared as described in section 2.7, and a solvent exchange was performed see appendix section A.

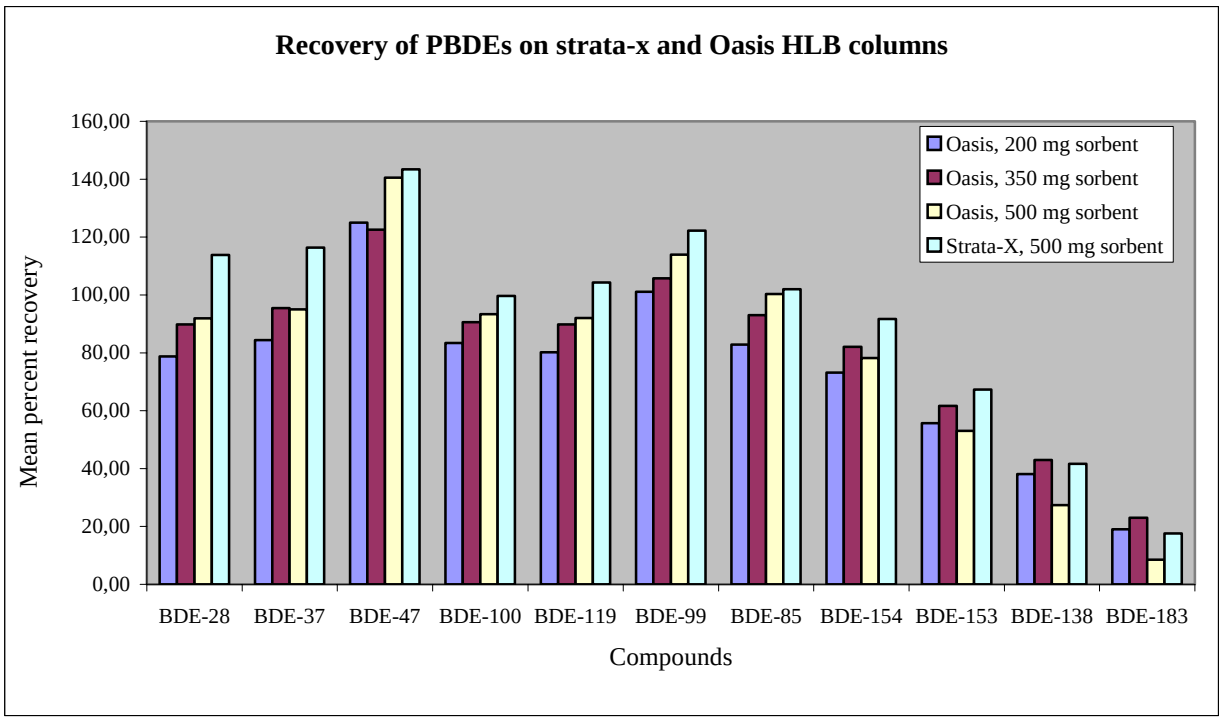

Figure 8. Mean percent recovery of PBDEs spiked in horse serum (n=4) obtained on Oasis HLB columns with different sorbent amounts, and Strata-x columns.

Figure 8 demonstrates the effect of different sorbent amounts. As can be seen, the recovery increases with increasing sorbent amounts for the PBDEs, the same trend was also seen with PCBs and phenols. High recoveries are obtained for BDE-47 and BDE-99 because of native concentrations in the horse serum or contribution from laboratory air. The highest overall recovery were obtained with the Strata-X columns, but the small particle size (33µm compared to Oasis HLB 60µm) caused pressure problems, especially with more viscous

samples such as plasma and these columns were therefore not found suitable for this application. The Oasis columns were then chosen, and the sorbent amount maximized. Custom-packed columns with 540 mg sorbent in 3 mL cartridges were purchased from waters. These columns were used in the method validation and in the following application.

3.3 Optimization of clean-up

In human serum there is about 0.5% lipids, but breast milk consists of approximately 3-5% lipids [56]. A column had to be designed for the removal of at least 3% lipids in 5 g samples of serum or milk (0.15 g lipids). Previously, columns filled with 1 g of sulphuric acid-silica (1:3 v/w) and 1.5 g of sodium sulphate had been used. Investigation of these columns revealed that they were only able to remove about 1.5% lipids. The bed volume was then maximized using 1.6 g of sulphuric acid-silica (1:3 v/w) and 1 g of sodium sulphate, the columns were now able to remove about 3% lipids. The effect of dividing the column into layers was then investigated. By creating a double-layer column as seen in Figure 4, up to 4% lipids were removed. The use of a more acidic sulphuric acid-silica (1:2 v/w) was also investigated, but was not found to be more efficient. Dividing the column into more than two layers did not improve the columns efficiency. It was found that an additional 0.1 g layer of activated silica on top of the columns provided cleaner extracts, due to the removal of cholesterol [57], this was applied to the columns used for clean-up of milk samples. The loss of PBDEs on the sulphuric acid-silica columns was investigated and the overall recovery was about 96%, so there was no considerable loss at this step.

The use of aminopropyl columns for the removal of lipids was also considered. The load limit on 500 mg aminopropyl columns was found to be approximately 10 mg of total lipid [58], and therefore these columns were not chosen.

3.4 Validation

3.4.1 Evaluation of internal standards

All PCBs were quantified using ^{13}C-ISTDs. For the PBDEs this was not an option when operating the gas chromatograph in NCI mode, so several BDE-congeners were investigated as potential ISTDs. Some of the criteria used when choosing the ISTDs were chemical structure and retention time. BDE-18 (tri-BDE) was found to the most suitable ISTD for BDE-28 (tri-BDE) and BDE-37 (tri-BDE). For BDE-47, which is a tetra-BDE, both BDE-51 (tetra-BDE) and BDE-77 (tetra-BDE) were examined as ISTDs. Good results were found with both BDE-51 and BDE-77, but BDE-51 was chosen because of the closeness in retention times. The tetra-BDE-77 and penta-BDE-103 were evaluated as ISTDs for BDE-99 and BDE-100, which both are penta-BDEs. BDE-103 were found to be the most suitable, being a penta-BDE and closest in retention time. For BDE-85 and BDE-119 which both are penta-BDEs, BDE-103 (penta-BDE) was found to be the most suitable ISTD. For the hexa-BDEs, BDE-153 and BDE-154, three ISTDs were evaluated, BDE-77 (tetra-BDE), BDE-103 (penta-BDE) and BDE-156 (hexa-BDE). All three BDEs were found suitable, with BDE-156 being closest in retention time and chemical structure, but less reproducible results were obtained with this compound compared to BDE-77 and BDE-103. The “second best” ISTD was therefore chosen, that being BDE-103 which was closer in retention time than BDE-77. BDE-183 is a hepta-BDE, and BDE-77 and BDE-181 (hepta-BDE) were evaluated as ISTDs for this congener. BDE-181 was found to be the most suitable ISTD.

BDE-77 and brominated biphenyl-77 (BB-77) has previously been used as ISTDs for all PBDEs [19], and BDE-77 was therefore used for comparison when evaluating new ISTDs.

All the ISTDs used in analysis of PCBs, PBDEs and phenols are listed in appendix section C.

3.4.2 Linearity

3.4.2.1 Calibration linearity

The linearity of the calibration curves was examined by the correlation coefficient, r. The area ratio between the analyte and ISTD was plotted against the corresponding concentration ratio. The standards were injected three times during an analysis sequence. The first and last injection was used in the calibration curve. Eight levels were used in the calibration curves for PBDEs and phenols, for the PCBs ten levels were used, the ranges are listed in section 2.3.

Because of the wide concentration range, two calibration curves were designed for each compound group, one in the low concentration range and the other in the high concentration range, each with six levels. Linear fit was used in all calibration curves except for the pesticide HCB and the phenols in the high concentration range, where a quadratic fit was used. All correlation values were in the range 0.989-1.000, with the lowest value accounting for the phenol PeBP.

3.4.2.2 Method linearity

The method linearity was examined by plotting the concentration of POPs found in the spiked samples against the concentration added. The native concentration of POPs in the horse serum was calculated, and this value was subtracted from the concentrations found in the spiked samples. Both the results from the initial validation and the second round when investigating the intermediate precision (section 2.8) were incorporated when evaluating the linearity. Regression results are presented in Table 8.

Because of high native concentrations of some of the PCBs (section 3.4.8) in the horse serum, the levels I, II and in some cases also level III could not be accurately quantified, and these compounds are therefore not listed in Table 8.

Table 8. Regression data obtained from the spiked validation samples (n=21).

Compounds	Slope (a)	Intercept (b)	Correlation coefficient (r)
BDE-28	0.8303	4.7361	0.9993
BDE-37	0.7657	5.0026	0.9996
BDE-47	0.6192	9.4487	0.9968
BDE-85	0.9190	2.7468	0.9999
BDE-99	0.8101	4.1520	0.9999
BDE-100	0.7857	4.6235	0.9998
BDE-119	0.8141	4.2840	0.9994
BDE-138	0.8554	3.5654	0.9997
BDE-153	0.9207	0.3592	0.9999
BDE-154	0.9005	1.3140	1.0000
BDE-183	0.9904	-2.3724	0.9998
BDE-209	0.9171	79.6490	0.9995
PeBP	1.1295	-4.6157	0.9994
TBBP-A	1.1749	-4.2801	0.9998

Table 8. Continued

Compounds	Slope (a)	Intercept (b)	Correlation coefficient (r)
HCB	0.9528	-0.5882	0.9999
OCS	0.9844	-1.5305	1.0000
CB-66	1.1043	-28.5480	0.9981
CB-81	1.0195	10.5710	0.9995
CB-105	1.0202	-0.7571	1.0000
CB-114	0.9897	2.1158	0.9999
CB-123	1.0042	-3.4690	0.9996
CB-128	0.9542	-1.8163	1.0000
CB-156	0.9934	3.4345	1.0000
CB-157	0.9932	0.7285	1.0000
CB-167	0.9929	2.5612	1.0000
CB-170	1.0416	-1.6148	1.0000
CB-180	1.0212	-3.4718	0.9999
CB-183	0.8828	8.4833	0.9999
CB-187	0.8771	8.3379	0.9999
CB-189	1.0146	0.4028	0.9999
CB-194	1.0195	-1.6568	1.0000
CB-209	1.0110	-1.2677	1.0000

3.4.3 Accuracy and precision

The accuracy was determined as recovery relative to ISTDs. The accuracy and precision at the five spiking levels (Table 9) outlined in section 2.8 were calculated and the results are presented in Table 10. Corrections were made for the native concentrations in the horse serum.

Table 9. Concentrations in pg/g serum of POPs in the spiked validation samples.

Level	TriBP	PeBP	TBBP-A	PBDE	BDE-209	PCB	m-o PCB, HCB, OCS
I	0.6	1.2	2.4	1.2	12	3.36	3.0
II	1.2	2.4	4.8	2.4	24	6.72	6.0
III	3.0	6.0	12	6.0	60	15.12	15
IV	9.0	18	36	18	180	60.48	60
V	60	120	240	120	1200	302.4	300

The number of replicates at all levels was 4, except at level IV were only one replicate was used.

Table 10. Mean accuracies of the different POPs found in spiked horse serum from the validation study.

Compounds	Level	n	Accuracy (%)	RSD (%)	Compounds	Level	n	Accuracy (%)	RSD (%)
BDE-28	I	4	87	8.1	**BDE-37**	I	4	97	11
	II	4	102	8.0		II	4	98	1.5
	III	4	97	3.2		III	4	96	3.6
	IV	1	99			IV	1	90	
	V	4	82	8.8		V	4	83	8.1
BDE-47	I	4	64	4.4	**BDE-85**	I	4	101	3.0
	II	4	94	17		II	4	102	4.4
	III	4	99	6.5		III	4	100	1.4
	IV	1	89			IV	1	99	
	V	4	71	7.9		V	4	92	2.7
BDE-99	I	4	109	25	**BDE-100**	I	4	100	3.4
	II	4	112	4.5		II	4	101	3.3
	III	4	96	4.5		III	4	97	3.4
	IV	1	90			IV	1	88	
	V	4	84	2.2		V	4	81	1.5
BDE-119	I	4	95	3.4	**BDE-138**	I	4	85	8.8
	II	4	91	3.5		II	4	97	2.5
	III	4	94	3.5		III	4	93	6.2
	IV	1	95			IV	1	97	
	V	4	94	3.3		V	4	87	1.2
BDE-153	I	4	96	4.4	**BDE-154**	I	4	93	2.7
	II	4	99	2.8		II	4	97	4.6
	III	4	98	2.2		III	4	99	5.2
	IV	1	94			IV	1	95	
	V	4	96	3.0		V	4	90	0.6
BDE-183	I	4	101	2.0	**BDE-209**	I	4	108	56
	II	4	104	8.0		II	4	150	33
	III	4	98	7.4		III	4	103	13
	IV	1	90			IV	1	100	
	V	4	90	6.7		V	4	95	1.3

Table 10. Continued

Compounds	Level	n	Accuracy (%)	RSD (%)	Compounds	Level	n	Accuracy (%)	RSD (%)
HCB[b]	I	4	78	15	**OCS**	I	4	89	6.2
	II	4	62	14		II	4	100	10
	III	4	104	5.3		III	4	101	4.0
	IV	1	97			IV	1	98	
	V	4	97	1.9		V	4	102	3.2
CB-18[b]	I	4	n.a[a]		**CB-28**[b]	I	4	n.a	
	II	4	n.a			II	4	n.a	
	III	4	n.a			III	4	127	27
	IV	1	65			IV	1	97	
	V	4	88	11		V	4	97	3.7
CB-52[b]	I	4	n.a		**CB-66**[b]	I	4	105	15
	II	4	n.a			II	4	76	20
	III	4	107	77		III	4	84	14
	IV	1	98			IV	1	101	
	V	4	88	8.6		V	4	106	4.3
CB-74	I	4	n.a		**CB-81**	I	4	88	11
	II	4	n.a			II	4	103	10
	III	4	94	5.1		III	4	112	7.3
	IV	1	100			IV	1	113	
	V	4	100	3.6		V	4	105	1.7
CB-99	I	4	n.a		**CB-101**	I	4	n.a	
	II	4	n.a			II	4	n.a	
	III	4	97	11		III	4	90	34
	IV	1	106			IV	1	114	
	V	4	102	1.9		V	4	102	1.9
CB-105	I	4	121	25	**CB-110**	I	4	n.a	
	II	4	111	20		II	4	n.a	
	III	4	107	1.5		III	4	n.a	
	IV	1	103			IV	1	98	
	V	4	102	2.3		V	4	112	4.5

Table 10. Continued

Compound	Level	n	Accuracy (%)	RSD (%)	Compound	Level	n	Accuracy (%)	RSD (%)
CB-114	I	4	99	2.6	**CB-118**	I	4	n.a	
	II	4	102	4.3		II	4	122	38
	III	4	102	1.6		III	4	100	14
	IV	1	102			IV	1	102	
	V	4	101	1.1		V	4	102	0.3
CB-123	I	4	29	22	**CB-128**	I	4	97	16
	II	4	69	12		II	4	94	10
	III	4	87	3.0		III	4	94	3.3
	IV	1	106			IV	1	96	
	V	4	102	1.4		V	4	97	1.7
CB-138	I	4	n.a		**CB-153**	I	4	n.a	
	II	4	n.a			II	4	n.a	
	III	4	96	11		III	4	107	15
	IV	1	94			IV	1	104	
	V	4	96	1.4		V	4	103	0.6
CB-156	I	4	108	6.1	**CB-157**	I	4	78	3.5
	II	4	109	1.8		II	4	83	10
	III	4	105	1.6		III	4	98	1.1
	IV	1	102			IV	1	102	
	V	4	101	0.7		V	4	101	1.1
CB-167	I	4	104	5.6	**CB-170**	I	4	102	14
	II	4	106	4.2		II	4	105	5.0
	III	4	101	2.2		III	4	105	2.6
	IV	1	101			IV	1	103	
	V	4	100	0.3		V	4	104	0.6
CB-180	I	4	104	39	**CB-183**	I	4	106	16
	II	4	98	29		II	4	109	7.1
	III	4	103	4.4		III	4	105	1.5
	IV	1	102			IV	1	93	
	V	4	102	0.8		V	4	91	2.3

Table 10. Continued

Compounds	Level	n	Accuracy (%)	RSD (%)	Compounds	Level	n	Accuracy (%)	RSD (%)
CB-187	I	4	107	37	**CB-189**	I	4	79	3.8
	II	4	109	19		II	4	96	3.5
	III	4	106	7.5		III	4	100	0.4
	IV	1	92			IV	1	104	
	V	4	90	2.6		V	4	102	0.5
CB-194	I	4	87	1.9	**CB-209**	I	4	90	3.8
	II	4	97	2.4		II	4	97	2.5
	III	4	101	1.4		III	4	100	1.4
	IV	1	102			IV	1	101	
	V	4	102	0.5		V	4	102	0.7
PeBP	I	4	101	24	**TBBP-A**	I	4	99	15
	II	4	119	14		II	4	77	24
	III	4	108	7.7		III	4	123	21
	IV	1	99			IV	1	132	
	V	4	104	5.9		V	4	123	4.5

na[a] because of high native concentrations of the compounds in the horse serum and procedural blanks these levels could not be accurately quantified.
[b] These compounds were determined in EI mode, the other PCBs and OCS were determined in CI mode.

The accuracy of the PBDEs were in the range of 64-150% with the mean being 95%, the RSD were in the range of 0.6-56% with a mean RSD of 6.9%. The high RSD-values found at low levels can be explained by the native concentrations of PBDEs found in the horse serum. The main PBDE congeners found in the horse serum was BDE-47, BDE-99 and BDE-209 (section 3.4.8), and these congeners were the most difficult at low levels. The mean accuracy of the PCBs were in the range of 29-127%, with a mean accuracy of 99%, the RSD were in the range of 0.3-77% with mean RSD of 8.7%. Some of the PCBs were found in relatively high concentrations in the horse serum (section 3.4.8), causing trouble in the low concentration range. The accuracy of the organochlorine compounds HCB and OCS were in the range of 62-104% with the mean being 93%. The RSD were in the range of 1.9-15%, with mean RSD of 7.5%. The phenols accuracy was in the range of 77-132% with a mean accuracy of 109%, the RSD ranged from 4.5-24% and the mean was 15%. TriBP were also

investigated in the validation, but high levels of this compound in the horse serum and procedural blanks made the quantification difficult.

3.4.4 Recovery of internal standards

The recovery of the ISTDs was calculated against a GC-MS recovery standard (CB-207) added to the samples just before analysis. The recovery standard corrects for differences in the final sample extract volume, and variations in the injection volume. Equation 1 was used in calculations.

(1) $$\%\text{Recovery} = \frac{100 * C_{ISTD}(std) * C_r(s) * A_{ISTD}(s)}{C_r(std) * C_{ISTD}(s) * A_r(s) * \left(\frac{A_{ISTD}}{A_r}\right)_{std}}$$

Abbreviations: C: concentration, ISTD: Internal standard, std: standard solution, s: sample extract, A: area, r: recovery standard.

Recovery was calculated in all the validation samples (n=17). Recovery of the ISTDs used for PBDEs were in the range of 64-89% with an overall recovery of 79%. The RSD was in the range 5-15% with an overall RSD of 9%. TBCr and CtriBBP-A used as ISTD for the phenols had recoveries of 64% and 53% respectively, with RSD of 8% (TBCr) and 35% (CtriBBP-A). Recoveries of the PBDEs and phenols ISTDs are illustrated in Figure 9. The PCBs ISTDs had recoveries in the range 37-82%, with an overall recovery of 63%. The RSDs ranged from 5-23% with an overall RSD of 9%, Figure 10 illustrates the results.

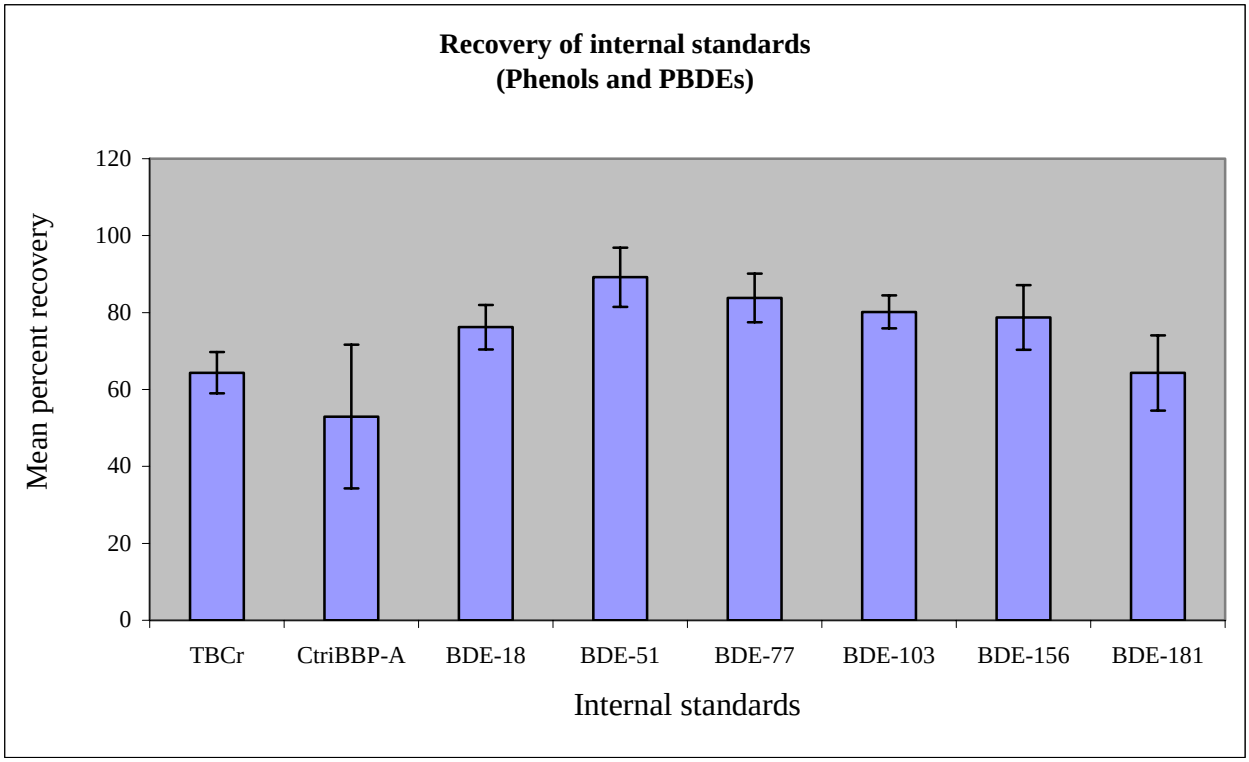

Figure 9. Recovery of internal standards used in the quantification of PBDEs and phenols. The relative standard deviation is shown as error bars (n=17).

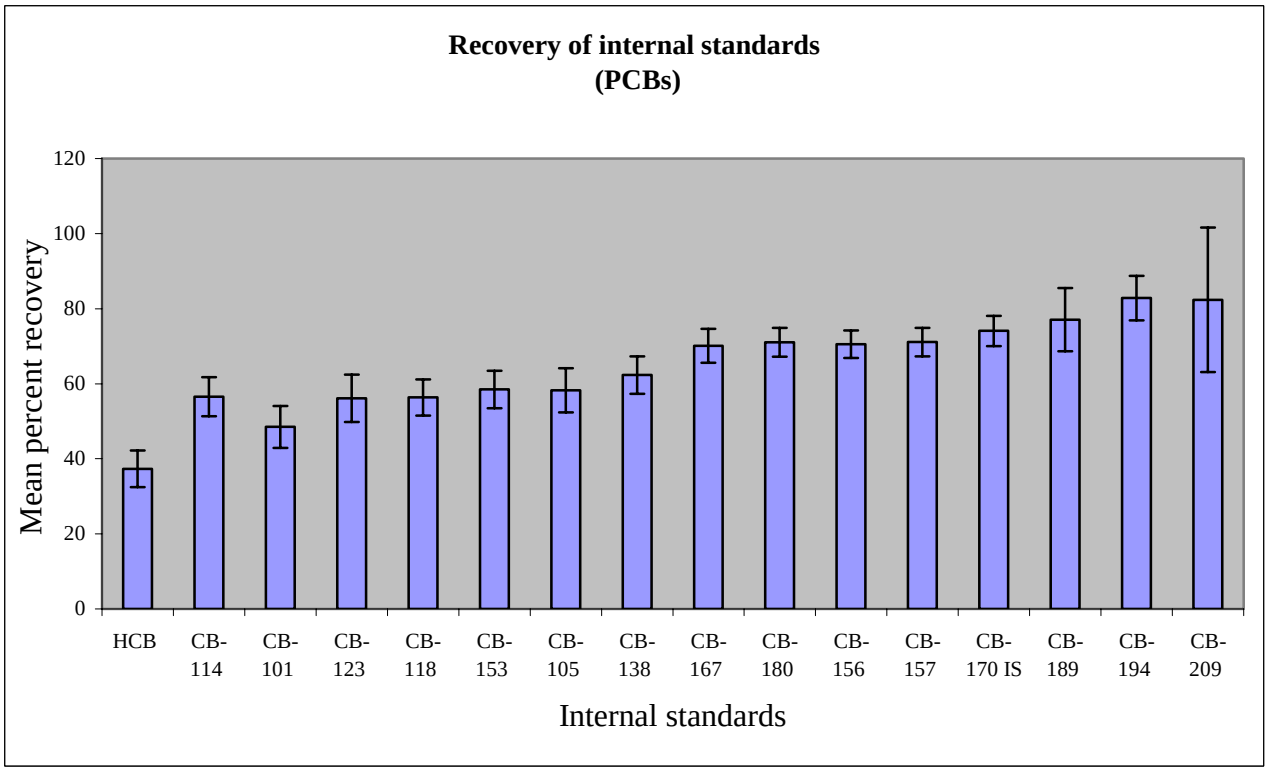

Figure 10. Recovery of internal standards used in the quantification of PCBs. The relative standard deviation is shown as error bars (n=17).

From Figure 10 it can be seen that the most volatile PCBs and the pesticide HCB have somewhat lower recoveries than the other congeners. This might be due to the solvent exchange performed on the spiking solutions. Because of this effect the solvent exchange will not be performed in future sample preparations and is not performed in the following application.

3.4.5 Intermediate precision

Two months after the initial validation, four new horse serum samples were spiked at level III (Table 9) extracted and analyzed as described in section 2.7. The PCBs were only analyzed in CI mode. The accuracy and repeatability was calculated and the results were compared with the initial validation. Corrections were made for the native concentration of POPs in the horse serum. Calibration curves used for quantification are described in section 2.3.

The mean accuracy found for the PBDEs at level III was 106% and the RSD was 4%. For the PCBs the accuracy was 98% and the RSD was 9%, CB-101 and CB-110 not included (due to high blank values). PeBP and TBBP-A had accuracies of 182% and 149% respectively, with RSD of 32.6% (PeBP) and 17.4% (TBBP-A). The organochlorine compound OCS had a mean accuracy of 93.1% and RSD of 4%. The difference in accuracy (relative to the initial validation), for the PBDEs were in the range of –1 to 18% with a mean difference of ± 9%, for the PCBs the difference was in the range of –32 to 22% with a mean difference of ± 8%. PeBP and TBBP-A had a difference of +74% and +26% respectively compared to the initial validation. OCS had a difference of ± 8%.

3.4.6 Selectivity and retention stability

Throughout the chromatographic analysis of the validation samples, the compounds retention time was highly stable and within ± 0.1 minutes. All compounds were baseline separated except CB-138, which were eluted between two other compounds (CB-163 and CB-164). Little interference was observed from matrix components. A chromatogram of a spiked horse serum sample at level III, for the PBDEs, is shown in Figure 11.

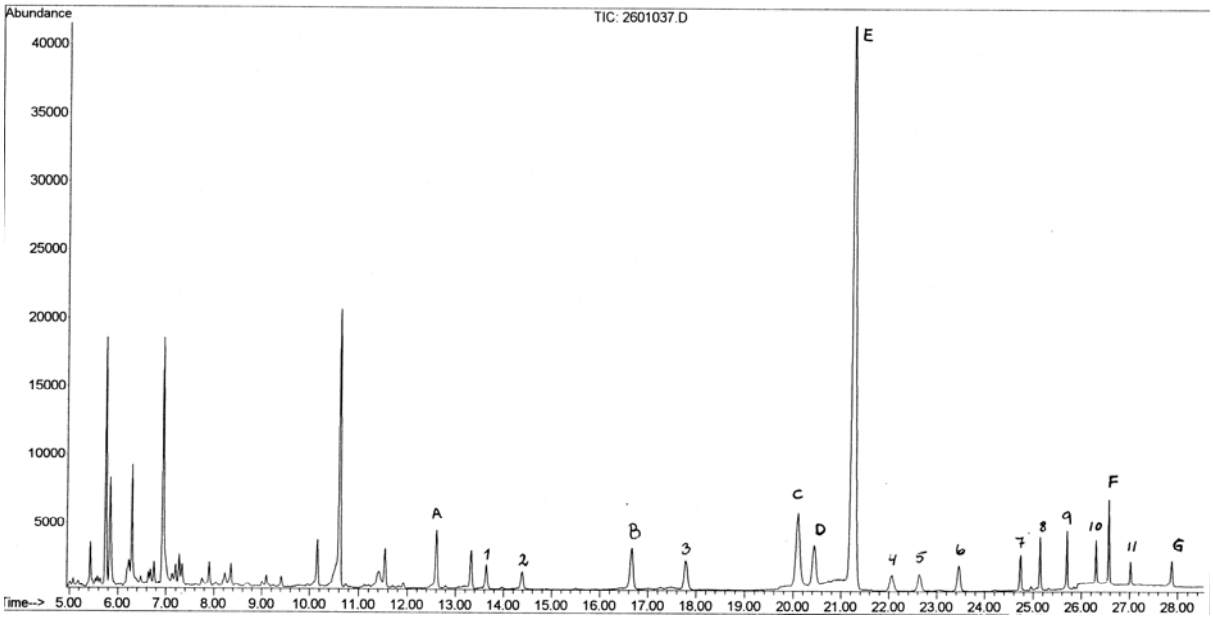

Figure 11. Chromatogram of level III (PBDEs) spiked in a horse serum sample. The numbers and letters corresponds to the following compounds: 1) BDE-28, 2) BDE-37, 3) BDE-47, 4) BDE-100, 5) BDE-119, 6) BDE-99, 7) BDE-85, 8) BDE-154, 9) BDE-153, 10) BDE-138, 11) BDE-183. A) BDE-18, B) BDE-51, C) C^{13}BDE-77, D) BDE-103, E) CB-207, F) BDE-156 and G) BDE-181.

3.4.7 Detection limits

The detection limits of POPs from the validation study were estimated by extrapolating the concentration that would result in a signal to noise ratio (S/N) of 3. The S/N ratio was measured in the lowest spiked level for all compounds except the PCBs analyzed in EI mode, where a non-spiked horse serum sample was used. Estimated limits of detection (LODs) are outlined in Table 11.

Table 11. Estimated LODs for compounds from the validation study.

Compounds	S/N	Concentration (pg/g serum)	LOD (pg/g serum)
BDE-28	8.0	1.29	0.5
BDE-37	6.7	1.44	0.6
BDE-47	9.8	5.79	1.8
BDE-85	11	1.24	0.3
BDE-99	14	2.48	0.5
BDE-100	5.5	1.18	0.6
BDE-119	4.5	1.09	0.7

Table 11. Continued

Compounds	S/N	Concentration (pg/g serum)	LOD (pg/g serum)
BDE-138	11	1.08	0.3
BDE-153	19	1.11	0.2
BDE-154	13	1.22	0.3
BDE-183	5.9	1.22	0.6
BDE-209	149	45.4	0.9
PeBP	7.0	1.71	0.7
TBBP-A	8.0	2.94	1.1
HCB[a]	23	19.0	2.5
CB-18[a]	5.2	97.0	56
CB-28[a]	90	418	14
CB-52[a]	12	96.0	24
CB-66[a]	16	87.0	16
OCS	18	2.70	0.5
CB-74	3.1	49.0	47
CB-81	39	3.92	0.3
CB-99	5.4	21.0	12
CB-101	61	116	5.7
CB-105	54	12.0	0.7
CB-110	15	70.0	14
CB-114	43	3.10	0.2
CB-118	477	44.0	0.3
CB-123	33	3.29	0.3
CB-128	18	7.02	1.2
CB-138	89	21.0	0.7
CB-153	234	35.0	0.4
CB-156	80	4.00	0.2
CB-157	78	2.54	0.1
CB-167	158	3.24	0.1
CB-170	112	4.38	0.1
CB-180	217	6.69	0.1
CB-183	132	4.36	0.1
CB-187	194	5.87	0.1

Table 11. Continued

Compounds	S/N	Concentration (pg/g serum)	LOD (pg/g serum)
CB-189	38	2.37	0.2
CB-194	158	2.90	0.1
CB-139	139	3.04	0.1

[a] These compounds were determined in EI mode.

3.4.8 Native concentrations of POPs in horse serum used for validation

The native concentration of POPs in non-spiked serum samples was investigated. The results are illustrated in Figure 12 and 13. The level in Figure 12 and 13 illustrates the sum of native concentrations in the horse serum and contributions from air, sorbents etc., this additional contribution can be measured by procedural blanks. By evaluating and correcting for the procedural blanks it was found that the main contribution did not come from the serum, but from air or during the sample preparation. The congeners that were found to be present in considerable amounts in the horse serum when correcting for the procedural blank were CB-99, CB-114 and CB-138. A chromatogram of a procedural blank for PBDEs is shown in Figure 14.

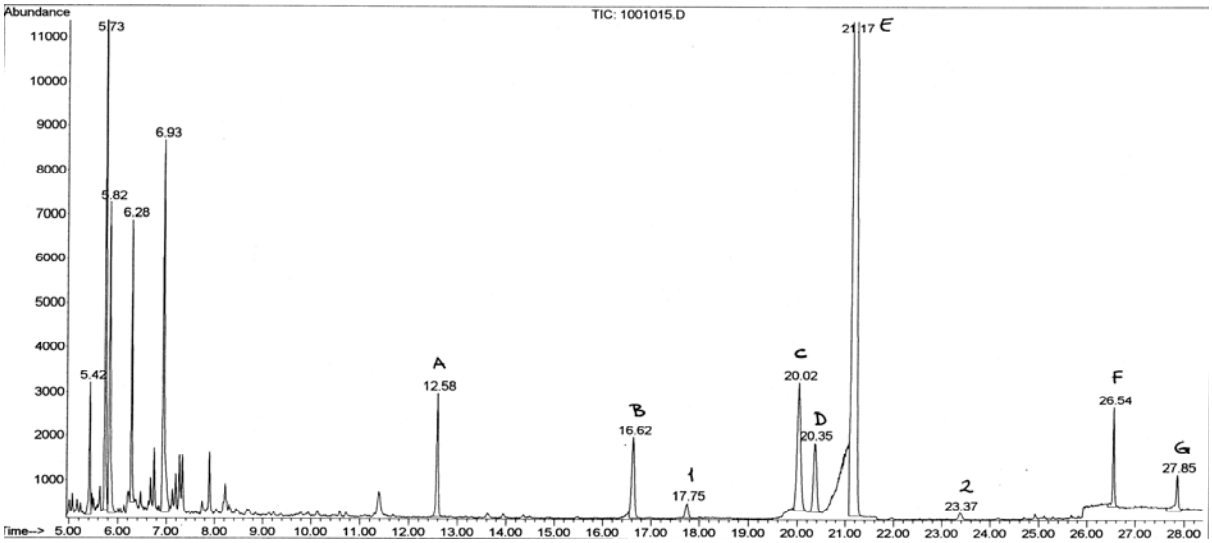

Figure 14. Chromatogram of PBDEs found in a procedural blank. The numbers and letters corresponds to the following compounds: 1) BDE-47, 2) BDE-99, A) BDE-18, B) BDE-51, C) C^{13}BDE-77, D) BDE-103, E) CB-207, F) BDE-156 and G) BDE-181.

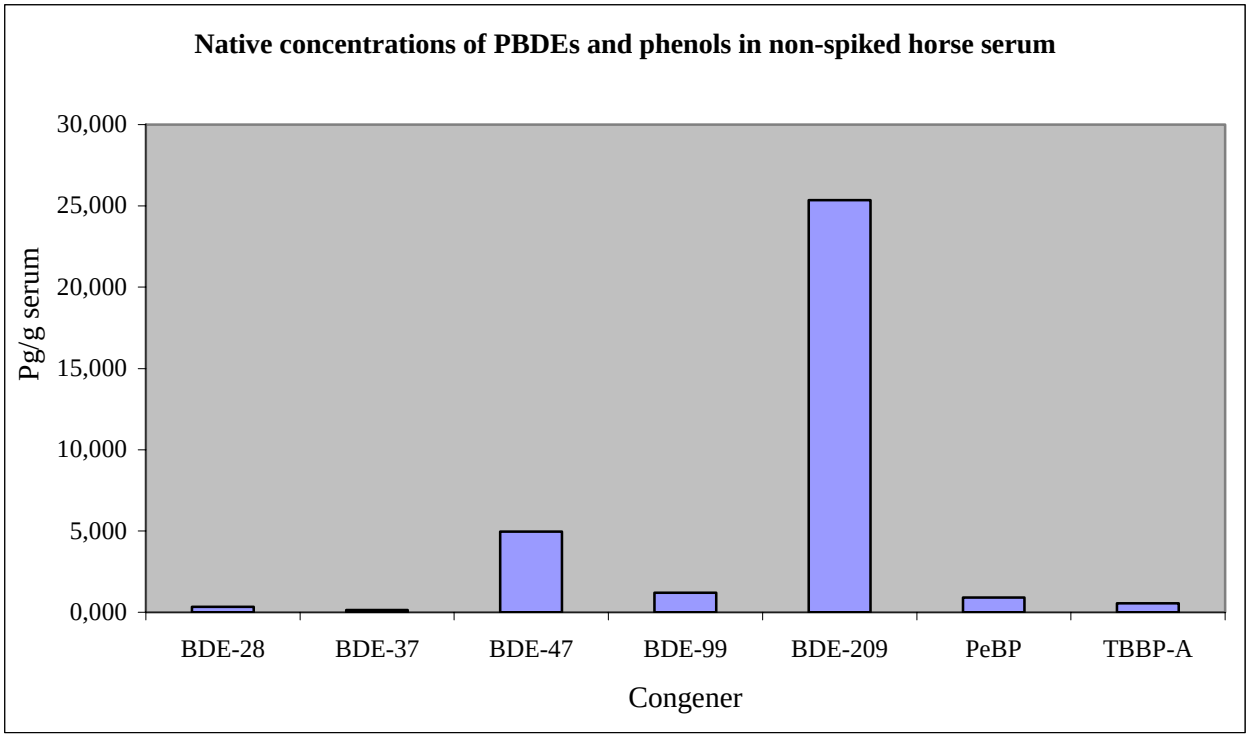

Figure 12. Native concentrations of PBDEs and phenols found in non-spiked horse serum (n=3).

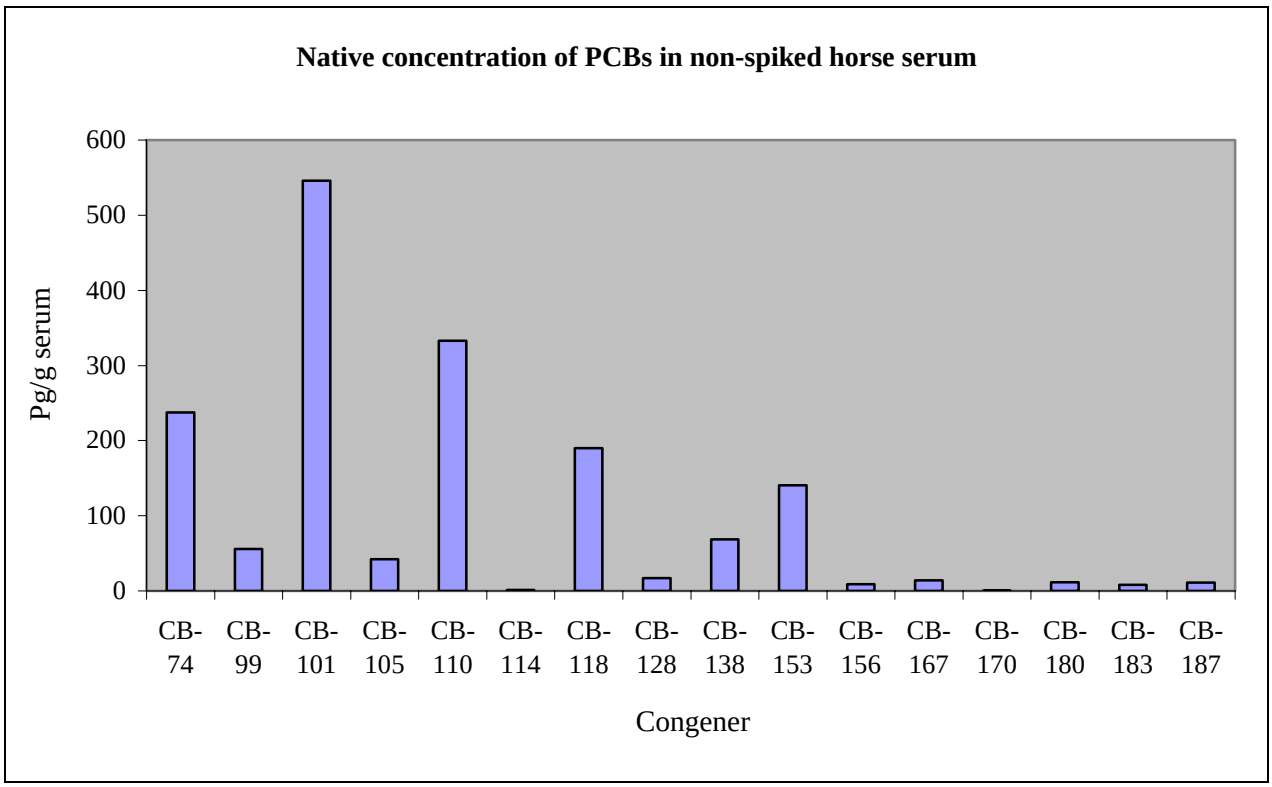

Figure 13. Native concentrations of PCBs found in non-spiked horse serum (n=3). The PCBs were determined in CI mode.

3.5 Time-trend and age trend study performed on pooled serum samples from Norway.

The method described above was used for the analysis of BFRs and PCBs in 21 pooled serum samples from the general population of Norway (section 2.2.2). In this study, the recovery of the ISTDs were in the range of 70-89% for the PBDEs with mean recovery of 80%, the RSDs were in the range of 8-17% with mean RSD of 12%. The recovery of the ISTDs used for the PCBs were in the range of 62-73% with mean recovery of 65%. The RSD was in the range of 8-11% with mean RSD of 9%. TBCr used as ISTD for TriBP and PeBP could not be correctly quantified because of a co-eluting compound. CtriBBP-A, the ISTD for TBBP-A had a recovery of 75%, with RSD of 16%. These recoveries were in close agreement with the results from the validation, except for CtriBBP-A where a higher recovery was obtained.

To assess the quality of the quantification in this study, samples form an interlaboratory comparison (section 2.2.3) were extracted and analyzed. Six PCB congeners (IUPAC No. 28, 118, 138, 153, 170 and 180) were calculated, and the results were compared with assigned values from the interlaboratory comparison. The accuracy of the control samples was in the range of 83-111%, with RSD in the range of 0.7-4%, except for CB-28, which gave very erroneous results. The reason for this is probably that the samples were analyzed in CI mode, which gives a very low response for this congener.

Concentrations of POPs and additional information concerning the serum pools are given in appendix Table D1. When looking at the serum pools from men in the age group 40-50 years, the serum concentration of seven PBDE congeners (IUPAC No. 28, 47, 99, 100, 153, 154 and 183) increased from 1977 (0.61 ng/g lipids) to 1998 (4.85 ng/g lipids), after 1998 there seems to be a small decrease or a stabilization and the concentration varies between 3.78 ng/g lipids and 4.62 ng/g lipids (Figure15). In the serum pool from 2002 elevated levels are observed, 5.75 ng/g lipids. The reason for this is unknown, but when comparing with men in the age group 25-59 years (3.32 ng/g lipids) in the age-trend study (Figure 17) the high levels found in the 2002 pool seems erroneous. The main PBDE congeners, found in all samples, were BDE-47 and BDE-153. The main PCB congeners were CB-138, CB-153 and CB-180. Closer studies of BDE-47 and BDE-153, revealed an increase in the relative amount of BDE-153 from the early 1990s, were the contribution from BDE-153 was 18% of total PBDE (sum of seven), to 2003 where the contribution was 38%. In the most recent serum pools, the contribution from BDE-47 and BDE-153 are approximately the same. The reason for this change in PBDE-pattern might be the change in use of technical mixtures of PBDE, (section 1), or differences in the accumulation potential of BDE-47 and BDE-153.

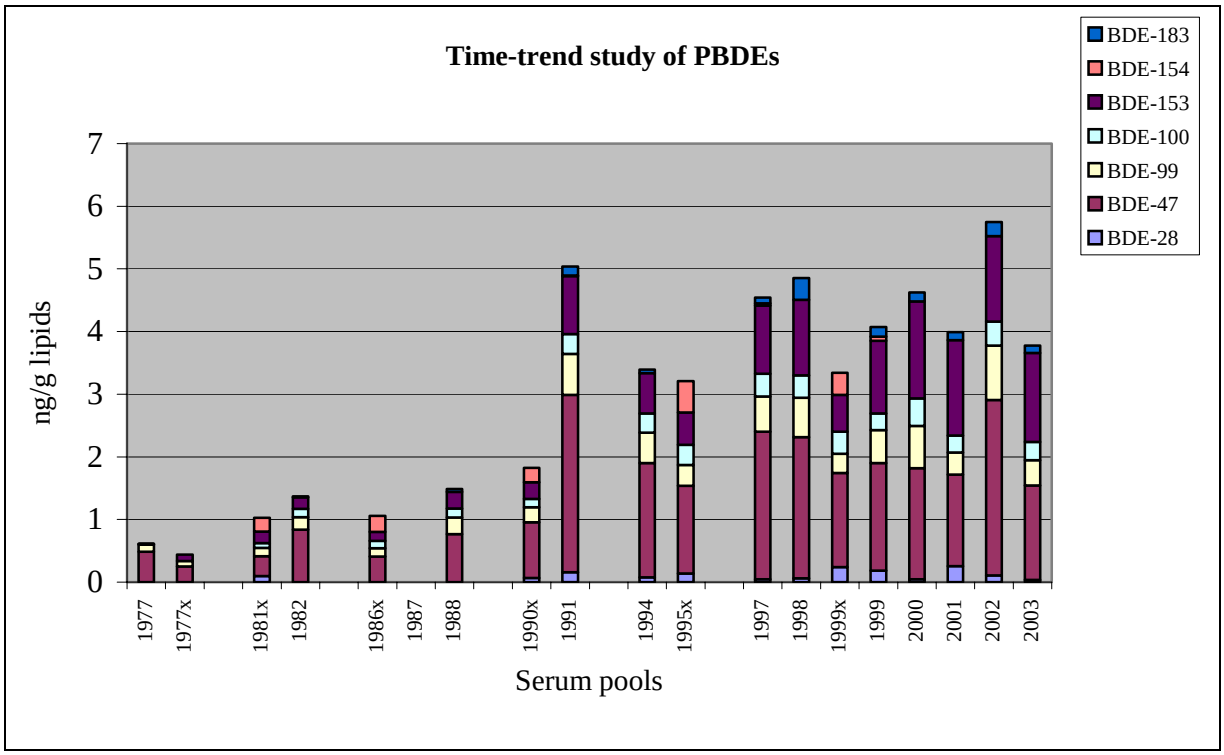

Figure 15. The sum of 7 PBDEs in ng/g lipids in pooled serum samples from Norway from 1977 to 2003. Incorporated in the figure are results from a similar study performed by Thomsen et al. [19], marked with an x. The samples from 1977 and 1977x are from the same pool, but the samples from 1999 and 1999x are from two different pools.

Elevated levels are also seen in the pool from 1991, the reason for this is unknown, but the pool might contain serum from several persons with high PBDE levels. In a study performed by Thomsen et al. [19], the sum of six PBDEs were used (IUPAC No. 28, 47, 99, 100, 153 and 154), see Figure 15. Thomsen et al. found BDE-154 in all sample pools except the oldest from 1977. In the present study BDE-154 was only detected in three sample pools (1991, 1997 and 1999). This can be explained by the fact that PBB-153 co-eluted with BDE-154 on the column used by Thomsen et al., this was not the case in the present study.

The time-trend of PCBs was also investigated using the same serum pools. Here the opposite trend was observed, with a decrease in the sum of five PCBs (IUPAC No. 101, 118, 138, 153 and 180) from 1977 to 2003, (665 ng/g lipids to 176 ng/g lipids) (Figure 16). Declining levels of PCB have previously been reported [20;21;59].

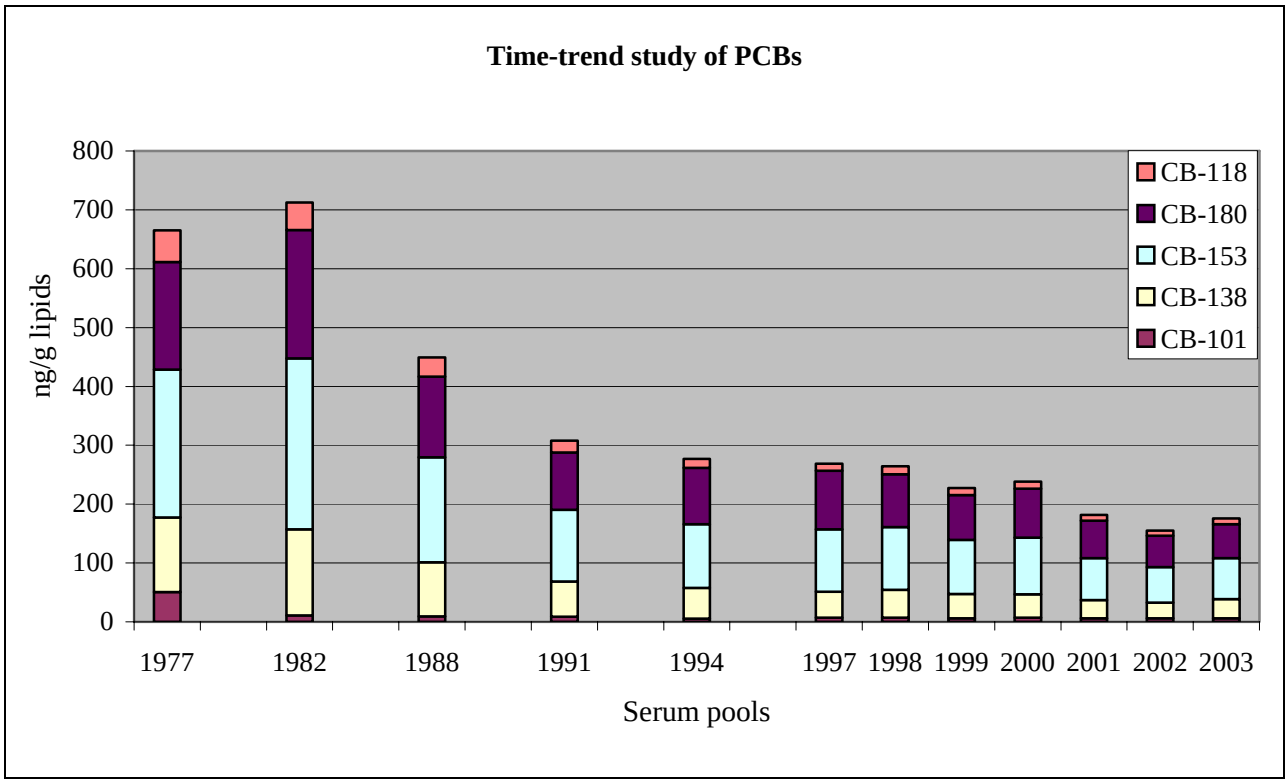

Figure 16. The sum of 5 PCBs in ng/g lipids in pooled serum samples from Norway from 1977 to 2003.

The level of CB-153 is about 46 times higher than the level of BDE-47 in the serum pool from 2003. This result is in agreement with pervious work, where the level of CB-153 was found to be 55 times higher than the level of BDE-47 in breast milk samples from Sweden, in 2000 [60].

An age-trend study of PCBs and PBDEs was performed on pooled serum samples from 2002. The PBDE concentration in the serum samples from the age groups 0-4 years (10.4 ng/g lipids) and 5-14 years (6.18 and 5.45 ng/g lipids for females and males respectively) was elevated compared to the other age groups, see Figure 17. As previously stated, food is regarded as a major source for human exposure to BFRs, and BFRs have been reported in breast milk [15;18;61] which is the main food source for small children. Prenatal exposure might also contribute to the elevated levels seen in infants, similar concentrations to that found in human milk have been reported in placenta [62]. Air and dust may also be an important contribution source. Young children are particularly vulnerable to contaminants found in dust as they are in close contact with floors and dusty surfaces, and small children often put hands and objects in their mouths. The exposure of PBDEs through inhalation has been estimated to be 7% of total exposure (93% from diet) [9]. Levels of PBDEs have been reported in dust from the US and Europe [63;64].

In the study by Thomsen et al. [19], the serum concentration was higher in the male pools, compared to female pools, for people above 25 years and older (Figure 17). This gender difference was much smaller in the present study. The reason for this is unknown.

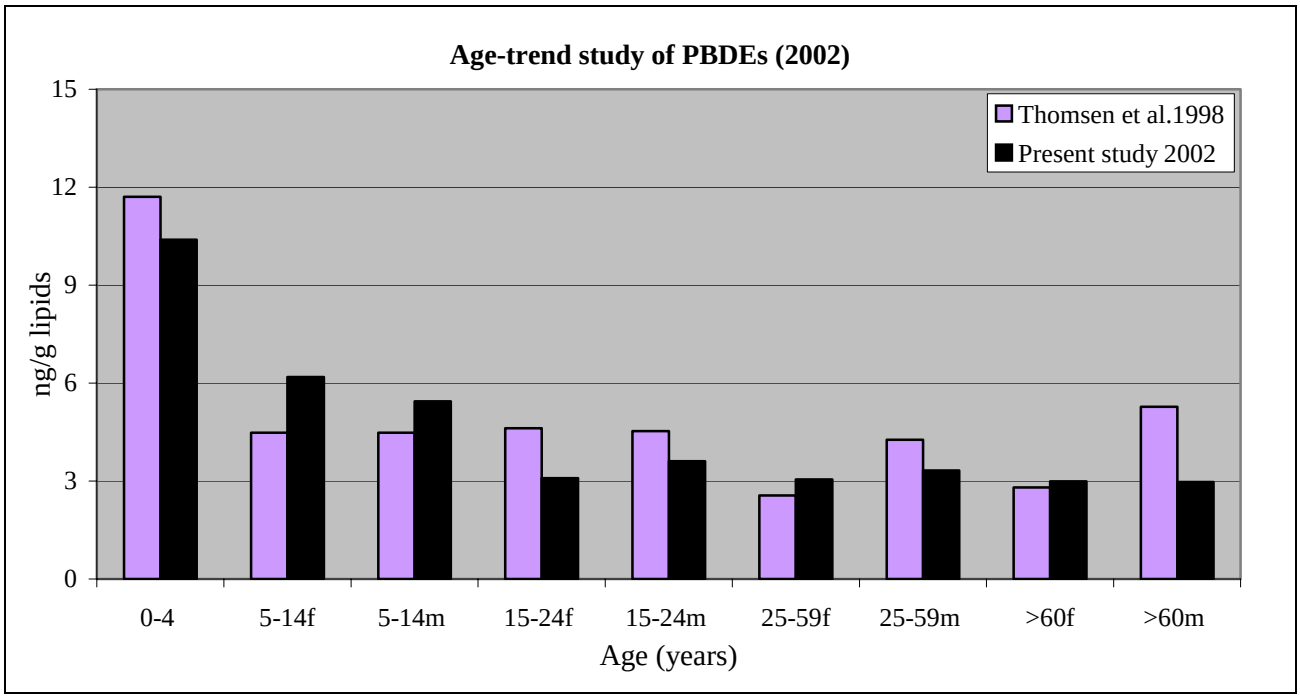

Figure 17. Two age-trend studies are compared. In 1998 the sum of six PBDEs was calculated, in 2002 the sum of seven PBDEs was used. In the age group 5-14 years only one pool was analyzed in 1998. Abbreviations: f; female, m; male.

The concentration of PBDEs in the pooled serum samples from 2002 have decreased compared to the study from 1998 (Figure 17), this is in agreement with the results from the time-trend study (Figure 15). The reason for the increase in concentration in the age group 5-14 years might be due to exposure during the 1990s, when the levels have been shown to be high (Figure 15). The children represented in the age group 0-4 years in the study by Thomsen et al. will in the present study be in the age group 5-14 years, this might be the reason for the increased levels.

In the age-trend study of PCBs, the sum of five PCBs increased from 97.5 ng/g lipid in the age group 0-4 years to 308 ng/g lipid and 353 ng/g lipid for women and men in the age group >60 respectively (Figure 18). These results are in agreement with the fact that PCBs accumulate over time.

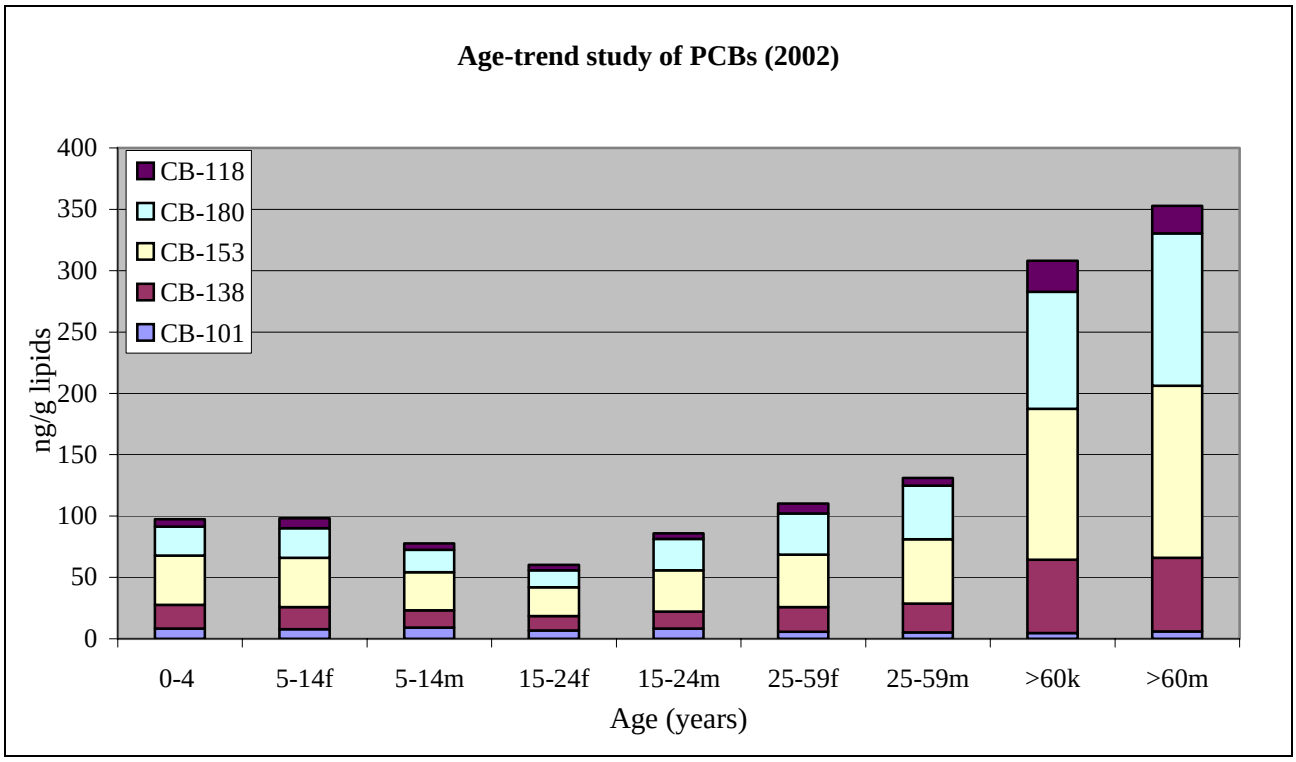

Figure 18. Age-trend study of the sum of five PCBs in pooled serum samples.

Chromatograms of a pooled serum sample (1998) and a procedural blank are illustrated in Figures 19 and 20. In the serum pool from 1998 (Figure 19) a peak is seen with retention time 21.36 minutes, this compound was identified as a nona-PCB and were found in all the serum pools.

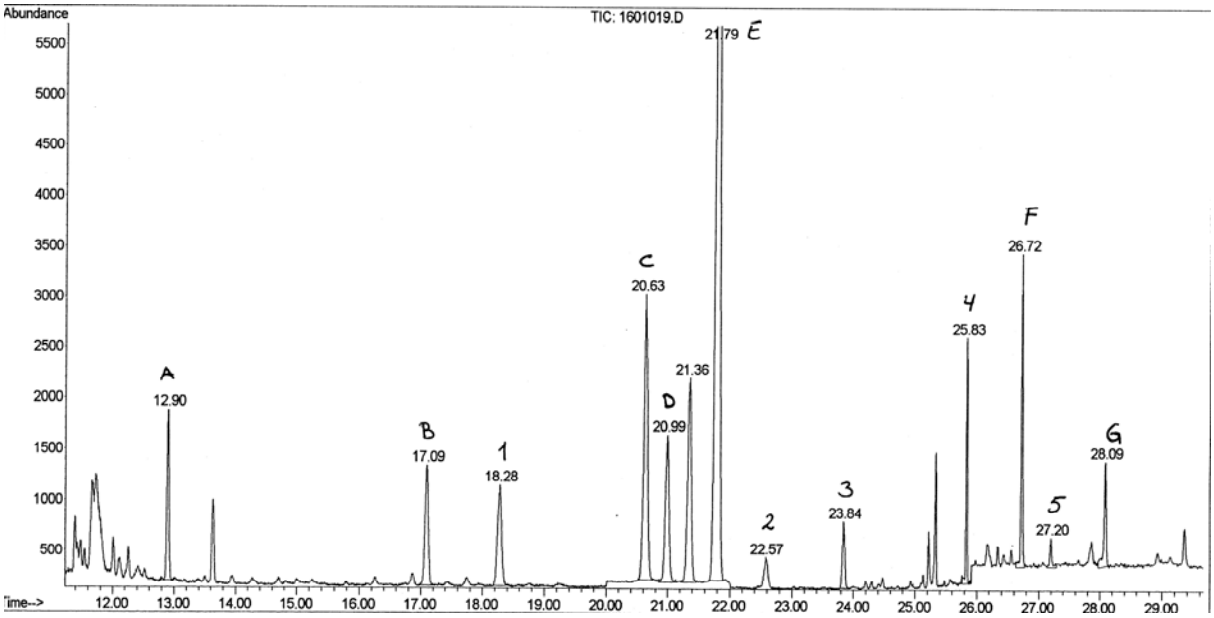

Figure 19. A chromatogram of a pooled serum sample from 1998. The numbers and letters corresponds to the following compounds: 1) BDE-47, 2) BDE-100, 3) BDE-99, 4) BDE-153, 5) BDE-

183, A) BDE-18, B) BDE-51, C) C^{13}BDE-77, D) BDE-103, E) CB-207, F) BDE-156 and G) BDE-181.

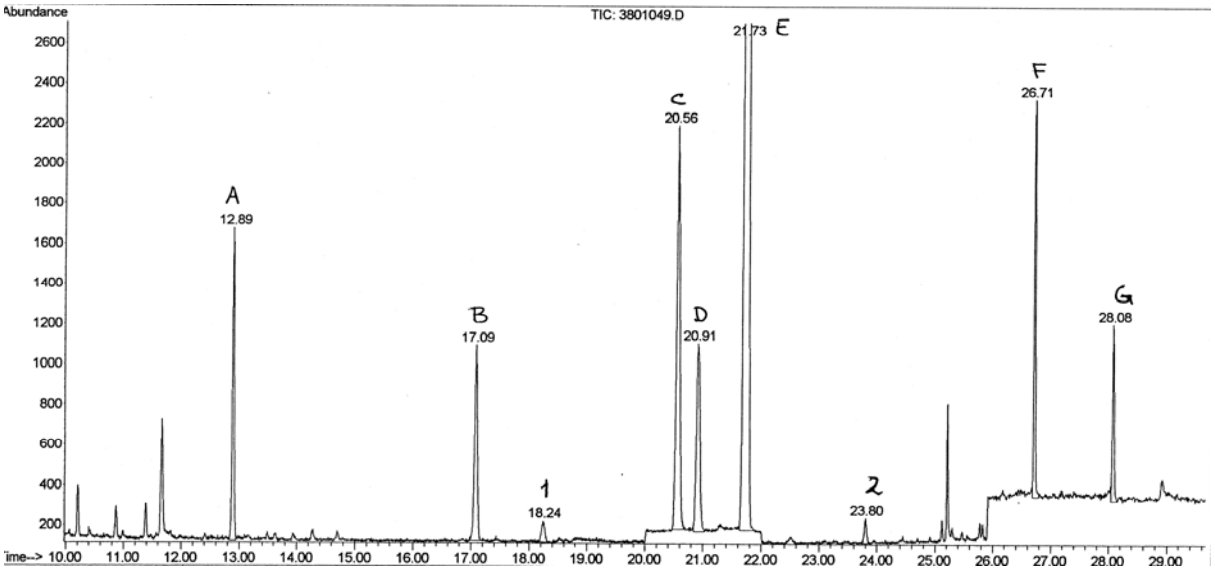

Figure 20. A chromatogram of a procedural blank extracted at the same time as the serum pools. 1) BDE-47, 2) BDE-99, A) BDE-18, B) BDE-51, C) C^{13}BDE-77, D) BDE-103, E) CB-207, F) BDE-156 and G) BDE-181.

TBBP-A was also determined in the serum pools. Prior to derivatization, the sample extracts were analyzed for native methylated derivatives of TBBP-A. Tetrabromobisphenol A dimethyl ether (methylated TBBP-A) was observed in all the serum pools. Further investigations revealed strong matrix effects in the analysis prior to derivatization, so the native concentrations could not be corrected for with the use of external standards. The following values given for TBBP-A are therefore the sum of TBBP-A (native) and methylated TBBP-A.

TBBP-A was found in all serum pools from 1982-2003. The concentration ranged from 0.01-0.66 ng/g lipids, with an average concentration of 0.40 ng/g lipids. Elevated levels were observed in the 1991 serum pool (1.01 ng/g lipids) and the 2000 serum pool (0.88 ng/g lipids) (Results not shown). In the different age group pools from 2002 the concentration of TBBP-A was in the range of 0.01-1.99 ng/g lipids. In one sample pool (men, 25-59 years) a very large peak was observed for TBBP-A, this peak could not be correctly quantified with the calibration curves used in this study.

BDE-209 was also detected in all serum pools. The sample pools from 1977 and 1982 are not included here, due to poor recovery. The reason for this is probably that these samples were more viscous and therefore difficult to work with due to old age and the fact that these

samples have been frozen and thawed several times. The concentration of BDE-209 in the serum pools from 1988 to 2003 was in the range of 5.28-36.7 ng/g lipids. The highest concentration of BDE-209 was found in the serum pool from 2000. The average concentration over the entire period was 11.0 ng/g lipids. In the age group pools from 2002 the concentration of BDE-209 was in the range 6.74-20.7 ng/g lipids. The average concentration in all the age group pools was 10.0 ng/g lipids. The highest concentration was observed in the age pool from female's age 15-24 years. Similar trends as that seen for the other PBDEs was not observed for BDE-209 or TBBP-A, but the two compounds were detected in almost all the samples. All values for BDE-209 and TBBP-A are given in appendix, Table D1.

4. Conclusion

The automated method developed for determination of POPs in human serum is fast compared to traditional manual SPE, simple and consumes low amounts of solvent compared to liquid-liquid extraction. The automatic unit is capable of working around the clock, and with the SPE method outlined in this study up to 24 serum samples can be extracted within 24 hours. For the additional clean-up step, which is less time consuming, up to 96 extracts can be cleaned-up within the same time period.

The method was validated, and the accuracy and precision was found satisfactory for most compounds. The phenols, BDE-209 and some of the PCBs were difficult, mainly due to high background levels (contributions from air, sorbents etc.). Detection limits were obtained in the range of 0.2-1.8 pg/g serum for the PBDEs and phenols, the PCB and OCs had LODs in the range of 0.1-56 pg/g serum. The linearity of the method was determined and correlation factors (r) of 0.9981 or better were found.

The method has been used to investigate the levels of PBDEs and PCBs in 21 pooled serum samples form the general Norwegian population and was found suitable for this application.

This fast and simple multi-method enables us to perform cost-effective, large-scale analysis-series, which are of utmost importance with respect to further investigations on POPs related to e.g. human exposure characterizations or possible health effects.

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6. APPENDIX

A. Solvent exchange

The presence of nonpolar solvents, used in the spiking solutions, was found to reduce the recovery of the polybrominated diphenyl ethers (PBDEs), especially the most lipophilic compounds. A change in solvent to a more polar solvent was therefore performed.

Procedure

Empty tubes (15 mL, screw cap) were spiked with internal standard solutions (total volume 150 μL). A solvent exchange was performed by adding 200 μL of ethanol to each tube, whirlmixing and carefully evaporating on a labline to a total volume of 200 μL. Samples were then prepared according to section 2.7.

Results/discussion

The solvent exchange resulted in an increase in recovery of about 10% for the PBDEs, Figure A1.

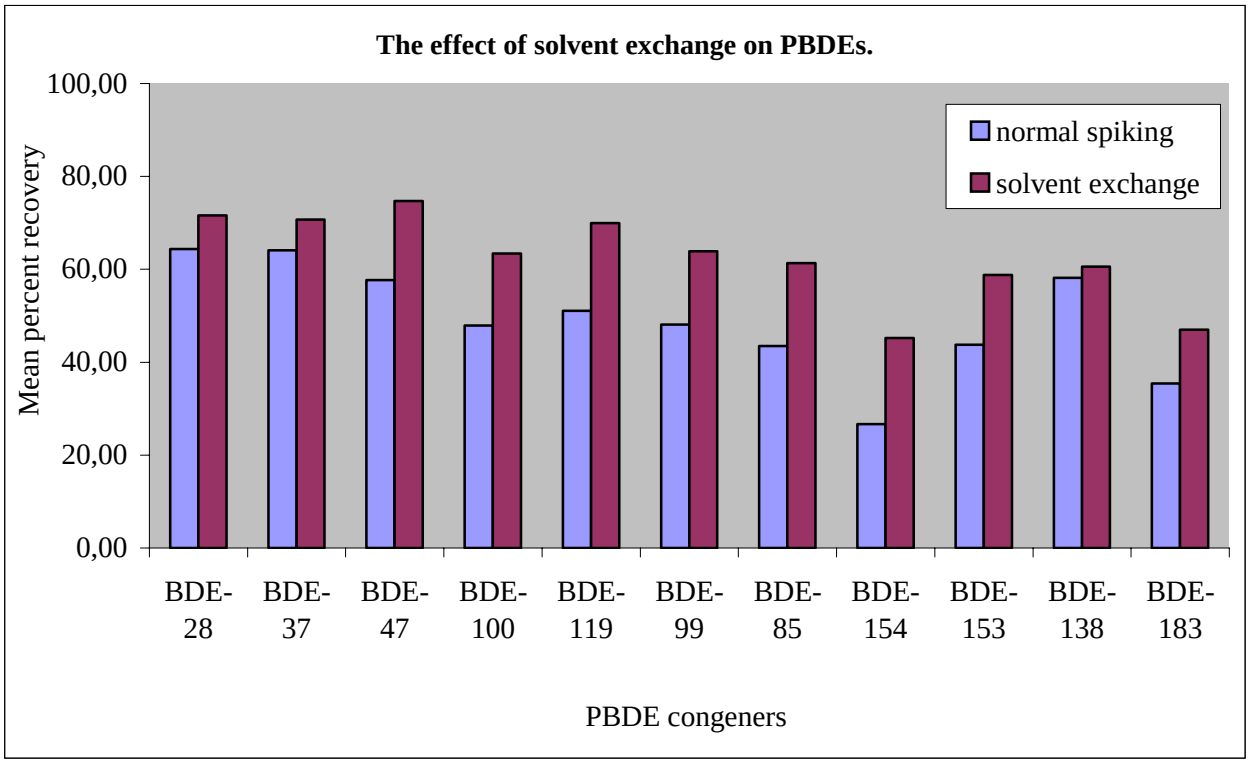

Figure A1. The effect of performing a solvent exchange is illustrated for PBDEs (n=4).

The solvent exchange was therefore applied during method development and method validation.

After the validation, section 3.4, it was concluded that the solvent exchange did not have a positive effect on the PCBs. Volatile compounds were lost during evaporation, section 3.4.4.

A solvent exchange was therefore not performed in the time-trend and age-trend study of POPs, section 3.5.

Since no solvent exchange were performed on the samples from the time-trend and age-trend study, several sonication steps were incorporated in the sample preparation to improve the mixing of the ISTD solutions with the serum.

B. Spiking solutions / Internal standards

All spiking solutions are listed in section 2.3. Spiking levels used in the method validation are listed in Table 9. Internal standard solutions added to the serum pools are outlined in Table B1.

Table B1. ISTDs added to the serum pools.

Volume added	Internal standards	Concentration
30 μL	PBDEs	2.5 pg/μL, 25 pg/μL BDE-209
30 μL	Phenols	1 pg/μL TBCr, 3 pg/μL CtriBBP-A
50 μL	PCBs	20 pg/μL
40 μL	Mono-ortho PCBs	5 pg/μL

Total volume added to each sample: 150 μL.

C. Internal standards

An overview of internal standards used in all experiments is listed in Table C1.

Table C1. An overview of compounds and their respective internal standards.

Compounds	**Internal standards (PCB, OCs)**	**Compounds**	**Internal standards (PBDE)**	**Compounds**	**Internal standards (Phenols)**
HCB	*^{13}C-HCB*	BDE-28 BDE-37	*BDE-18*	TriBP PeBP	*TBCr*
CB-18 CB-28	*^{13}C-CB-28*	BDE-47	*BDE-51*	TBBP-A	*CtriBBP-A*
CB-52	*^{13}C-CB-52*	BDE-99	*BDE-77*		
OCS CB-66 CB-74 CB-81 CB-99 CB-101 CB-110	*^{13}C-CB-101*	BDE-85 BDE-100 BDE-119 BDE-153 BDE-154	*BDE-103*		
CB-105	*^{13}C-CB-105*	BDE-138	*BDE-156*		
CB-114	*^{13}C-CB-114*	BDE-183	*BDE-181*		
CB-118	*^{13}C-CB-118*	BDE-209	*^{13}C-BDE-209*		
CB-123	*^{13}C-CB-123*				
CB-138	*^{13}C-CB-138*				
CB-153	*^{13}C-CB-153*				
CB-156	*^{13}C-CB-156*				
CB-157	*^{13}C-CB-157*				
CB-128 CB-167	*^{13}C-CB-167*				
CB-170	*^{13}C-CB-170*				
CB-180 CB-183 CB-187	*^{13}C-CB-180*				
CB-189	*^{13}C-CB-189*				
CB-194	*^{13}C-CB-194*				
CB-209	*^{13}C-CB-209*				

D. Raw data from the time-trend and age-trend study.

The concentration of PBDEs, PCBs and TBBP-A found in the pooled serum samples, section 2.2.2, are listed in Table D1.

Table D1. Serum concentrations of PBDEs, PCBs and TBBP-A in ng/g lipids, pooled serum samples.

Serum pool	Fat %	N	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153	BDE-154	BDE-183	BDE-209	CB-28	CB-101	CB-118	CB-138	CB-153	CB-180	TBBP-A
1977	0.62	34	Nd	0.49	0.11	0.01	0.01	Nd	Nd	Nd	174	50.8	53.8	127	251	183	
1977x	0.62	34	Nd	0.25	0.09	Nd	0.10	Nd	-	-	-	-	-	-	-	-	
1981	0.67	17	0.10	0.32	0.13	0.08	0.18	Nd	Nd	-	-	-	-	-	-	-	Nd
1982	0.72	18	Nd	0.84	0.20	0.13	0.18	Nd	0.02	Nd	92.1	10.9	46.6	146	290	218	0.06
1986	0.74	24	Nd	0.41	0.13	0.12	0.14	Nd	Nd	-	-	-	-	-	-	-	0.44
1988	0.63	23	Nd	0.77	0.26	0.14	0.27	Nd	0.05	7.43	Nd	9.20	33.0	91.8	179	137	0.53
1990	0.68	20	0.07	0.89	0.24	0.13	0.27	Nd	Nd	-	-	-	-	-	-	-	0.42
1991	0.72	19	0.16	2.83	0.65	0.32	0.92	0.01	0.14	5.28	Nd	8.51	19.9	60.1	122	97.5	1.01
1994	0.68	30	0.07	1.83	0.48	0.31	0.65	Nd	0.06	6.61	Nd	5.60	15.4	52.2	108	95.5	0.66
1995	0.69	19	0.14	1.40	0.33	0.32	0.52	Nd	Nd	-	-	-	-	-	-	-	0.59
1997	0.61	14	0.04	2.35	0.56	0.36	1.09	0.04	0.09	4.69	Nd	6.82	11.8	44.1	106	99.6	0.45
1998	0.66	20	0.06	2.25	0.63	0.36	1.20	Nd	0.34	15.0	Nd	6.91	13.4	47.3	107	89.7	0.16
1999	0.72	20	0.18	1.72	0.53	0.27	1.16	0.07	0.15	10.2	Nd	6.07	12.1	41.5	91.5	76.1	0.41
1999x	0.65	29	0.24	1.50	0.31	0.35	0.59	0.35	-	-	-	-	-	-	-	-	
2000	0.60	20	0.05	1.77	0.68	0.44	1.55	Nd	0.14	36.7	Nd	7.30	11.6	39.6	96.1	83.5	0.88
2001	0.62	20	0.25	1.46	0.35	0.27	1.52	Nd	0.13	6.18	Nd	5.77	9.63	31.2	71.0	63.9	0.06
2002	0.64	20	0.11	2.80	0.87	0.38	1.37	Nd	0.22	7.60	Nd	6.15	8.62	26.3	60.7	53.1	0.08
2003	0.73	20	0.03	1.51	0.40	0.29	1.42	Nd	0.12	10.1	Nd	6.08	10.1	32.7	69.4	57.5	0.06
0-4 years	0.51	29	0.08	4.57	2.27	1.02	1.67	0.09	0.06	8.70	Nd	8.29	6.08	19.4	40.1	23.6	0.19
5-14f years	0.55	20	0.09	2.71	1.27	0.70	1.38	0.07	0.20	11.7	Nd	7.86	7.91	18.1	40.1	24.2	0.02
5-14m years	0.54	20	0.08	2.18	0.76	0.49	1.85	Nd	0.05	10.3	Nd	9.10	5.21	14.2	31.0	18.2	0.01
15-24f years	0.57	20	0.08	1.30	0.39	0.28	0.95	Nd	0.11	20.7	Nd	6.75	4.50	11.7	23.4	13.8	0.15
15-24m years	0.47	20	0.08	1.32	0.41	0.21	1.46	Nd	0.12	6.74	Nd	8.45	4.65	13.7	33.6	25.5	0.44
25-59f years	0.67	20	0.04	1.56	0.35	0.30	0.77	Nd	0.02	7.64	Nd	5.79	7.96	20.1	42.7	33.6	0.10
25-59m years	0.61	20	0.09	1.25	0.34	0.20	1.28	Nd	0.18	6.83	Nd	5.16	6.10	23.5	52.4	43.9	Nd
>60f years	0.75	20	0.09	1.27	0.38	0.28	0.91	Nd	0.06	9.68	Nd	4.65	25.3	59.8	123	95.4	1.99
>60m years	0.69	20	0.09	1.18	0.28	0.29	1.09	Nd	0.06	7.39	Nd	6.00	22.4	60.0	140	124	0.28

Abbreviations: f, females; m, males; Nd, not detected; - not analyzed